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(71) Applicant: APPLIED PHYTOLOGICS, INC. [US/US]; 4110 North Freeway Boulevard, Sacramento, CA 95834–1219 (US).

(72) Inventors: VON WETTSTEIN, Diter; Apartment 4, 205 N.W. Anthony Street, Pullman, WA 99163 (US). HAGIE, Frank, E., Jr.; 2417 Hale Drive, Burlingame, CA 94010 (US).

(74) Agents: EVANS, Susan, T. et al.; Dehlinger & Associates, P.O. Box 60850, Palo Alto, CA 94306–0850 (US).

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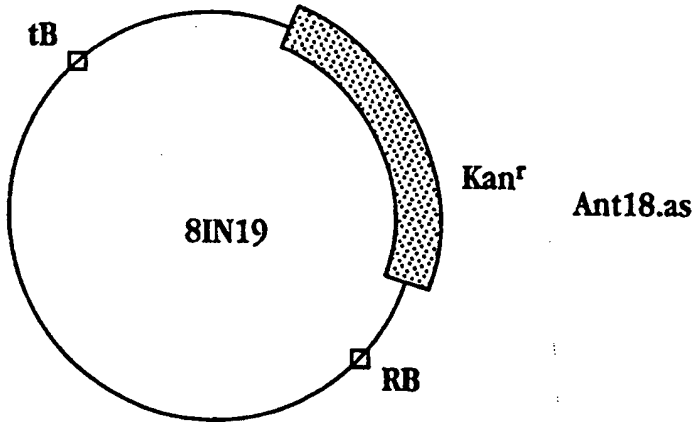
(54) Title: PATHOGEN–RESISTANT MONOCOT PLANTS AND METHOD

(57) Abstract

A method of producing resistance to a plant microbial pathogen in the seeds of a monocot plant is disclosed. A plant is stably transformed with a chimeric gene having (i) a transcriptional regulatory region induced during seed development, and (ii) an inhibitory gene sequence operably linked to the transcriptional regulatory region for induction of an inhibitory transcript RNA during seed development. The biological action of the transcript RNA, at the levels induced, leads to an accumulation of the secondary intermediate metabolite at levels toxic to the microbial pathogen. Pathogen resistant plants and seeds from the method are also disclosed.



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PATHOGEN-RESISTANT MONOCOT PLANTS AND METHOD

Field of the Invention

The present invention relates to a method of producing pathogen resistant monocot plants, and to plants and seed compositions produced thereby.

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Background of the Invention

Microbial infection of monocot seeds, meaning seed infection due to a fungal, bacterial or viral agent, is a significant agricultural problem, often resulting in pronounced loss of seed quality and useability.

Monocot seeds are susceptible to pathogen, *e.g.*, fungal infection, both in the field and as stored seeds. Although some fungi infect seeds under both field and storage conditions, the main genera of fungi falling into these two classes is divided. Field fungi invade seeds during their development on plants in the field or following harvesting while the plants are standing in the field. Field fungi require a high moisture content for growth. Thus, periods of high rainfall at harvest can result in extensive grain deterioration. The main fungal species associated with crops such as wheat or barley in the field are *Alternaria, Fusarium*, and *Helminthosporium* species, while *Fusarium moniliforme* primarily attacks corn. Other field fungi associated with grain crops include *Cladosporium* and *Trichoderma* ssp.

Species of storage fungi that may be present on the seeds and which develop during wet storage are mostly, although not strictly, of the genera *Aspergillus* and *Penicillium*, and infest seeds under storage or germination conditions. Some of the major deleterious effects of storage fungi on seeds are to (i) decrease viability, (ii) cause discoloration, (iii) produce mycotoxins, (iv) cause heat production, and (v) develop mustiness and caking.

Current control measures employed against plant fungal diseases include breeding cultivars for improved fungal resistance, using improved planting or growing conditions that reduce risk of infection, and treating crops or seeds with anti-fungal agents.

These approaches have various problems that limit their effectiveness. For example, genetic resistance is often lacking in the desired commercial cultivars, while improved planting and growth conditions, such as canopy management and reduced planting densities, are labor intensive and often of limited efficacy. The application of chemical fungicides for controlling plant fungal diseases may be expensive and have associated health risks and environmental restrictions. Specific methods for controlling fungal infection in seeds may involve seed soak and seed drench practices. However, such practices have led to resistant strains, and loss of effectiveness of the fungicidal agent in use. Further, fungi such as *Fusarium* cause diseases which are difficult to control and result in significant reductions in yield and grain quality. *Fusarium* is also the source of toxins produced in stored grains.

The continued economic toll taken by microbial pathogens suggests a need to develop new, more effective approaches for preventing microbial infection, particularly fungal infection of seed crops, such as barley, wheat, corn, and rice. Additionally, these requirements should be met without significant adverse side effects to the plant or environment, and without seriously restricting planting or growth conditions, or requiring expensive chemical treatment of either growing plants or harvested seeds.

Summary of the Invention

The present invention is based on the discovery that the buildup of certain secondary intermediate metabolites in seeds is effective to block microbial, *e.g.*, fungal, infection of the seeds. By placing under the control of a seed-inducible promoter, an inhibitory gene sequence whose expression results in a buildup of the metabolite in seeds, it is possible to specifically induce pathogen resistance in seeds, or within certain seed tissues, such as the seed testa layer, without significantly interfering with plant growth and development and other phenotypic traits in the plant as a whole. That is, the transformed monocot plants produce a secondary metabolite at levels toxic to a given pathogen in seeds or localized seed tissue only, allowing the plant itself to develop normally and at normal seed production levels.

In practicing the method of the invention, there is first identified an intermediate secondary metabolite that can be shown to inhibit infection of the plant pathogen in monocot seeds, at above-normal levels of the metabolite. From this, the gene that encodes an enzyme that normally converts the intermediate secondary metabolite to another metabolite that is less toxic to the pathogen is also identified. The plant is then stably transformed with a chimeric gene having (i) a transcriptional regulatory region induced during seed development, and (ii) an inhibitory gene sequence operably

linked to the transcriptional regulatory region for induction of an inhibitory transcript RNA during seed development. The biological action of the transcript RNA, at the levels induced, leads to an accumulation of the secondary intermediate metabolite at levels toxic to the microbial pathogen.

The inhibitory gene sequence is preferably one whose induction leads to the production of a transcript capable of sequence-specific hybridization with the mRNA transcript of a plant gene encoding an enzyme that normally converts the intermediate secondary metabolite to another metabolite that is less toxic to the pathogen. The transcription regulatory region is preferably specific for seed tissues, such as aleurone, endosperm, embryo, pericarp or testa-layer cells.

As an example, seed resistance to *Fusarium* can be achieved by stably transforming a plant with a gene whose transcript RNA blocks expression of the enzyme dihydroquercetin reductase (DHQR), to block conversion of dihydroquercetin (DHQ), which is shown herein to strongly inhibit *Fusarium* infection of seeds, to 2,3-*trans*-3,4-*cis*-leucocyanidin. The inhibitory gene sequence may produce an inhibitory transcript RNA capable, for example, of sequence-specific binding to the transcript of the structural gene of barley, corn or rice DHQR, whose sequence is identified by SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3, respectively.

Also disclosed is a monocotyledonous plant having enhanced resistance to a microbial pathogen. The plant is stably transformed with a chimeric gene having (i) a transcriptional regulatory region induced during seed development, and (ii) an inhibitory gene sequence operably linked to the transcriptional regulatory region for induction of an inhibitory transcript RNA during seed development. The biological action of the transcript RNA, at the levels induced, leads to an accumulation of a secondary intermediate metabolite at levels toxic to the microbial pathogen.

In a related aspect, the invention includes pathogen-resistant seeds obtained from the stably transformed plant.

These and other objects and features of the invention will become more fully apparent when the following detailed description of the invention is read in connection with the accompanying drawings.

Brief Description of the Figures

Figs. 1A-1F are HPLC profiles of monomeric flavonoids extracted from (A) Triumph, (B) *ant*18-159 barley, (C) *ant*13-152 barley, (D) *ant*17-148 barley; (E) DHQ standard; and (F) catechin standard;

Figs. 2A-C show flavonoid biosynthesis pathways in barley;

Figs. 3A-3D are computer-scanned images of barley (20 days after anthesis) infected *in vitro* at a *Fusarium culmorum* spore concentration of 4×10^5 spores/10 g kernels for one week (Fig. 3A = Triumph, Fig. 3B = *ant*13-152, Fig. 3C = *ant*17-148, and Fig. 3D *ant*18-159);

Figs. [redacted] a schematic representation illustrating the [redacted]ts of testa/pericarp extracts from proanthocyanidin free mutants and their mother varieties on growth of *Fusarium culmorum* on CZID plates incubated for 10 days;

Fig. 5 presents the reaction of dihydroflavonol-4-reductase (DFR, also referred to as dihydroquercetin reductase or DHQR), which reduces dihydroflavonols to 3,4-cis-leucoanthocyanidins;

Fig. 6 illustrates a difference PCR amplification method for identifying a testa-specific promoter in a monocot;

Figs. 7A and 7B show the sequences of the two Difference Products after three amplification cycles, designated RDA-1 (SEQ ID NO:6) and RDA-2 (SEQ ID NO:7);

Figs. 8 illustrates schematically (i) reverse transcription of the mRNAs with either oligo dT primers or primer 2 (oligonucleotide derived from the sequence of the Difference Product) and primer 1 (also derived from the sequence of the difference product), and (ii) PCR amplification of the obtained cDNA; and

Figs. 9A-9E illustrate construction of an exemplary chimeric DNA containing an anti-sense DHQR gene under the control of a seed-induced promoter, where H=*Hind*III, N=*Nco*I, E=*Eco*RI, B=*Bam*HI.

Detailed Description of the Invention

I. Definitions and Abbreviations

Unless indicated otherwise, the terms below have the following meaning:

"Monocot" or "monocotyledonous plant", refers to a an angiosperm (*i.e.*, a flowering plant) that has only a single cotyledon (seed leaf) that is formed during embryogenesis. Representative monocots include wheat, maize, barley, rice, millet, oats, rye, triticale, and sorghum.

"Plant microbial pathogen" refers to a fungal, bacterial, or viral pathogen that infects plants, including the seeds thereof, with a resulting pathogenic effect on the plant.

"Resistance to a plant microbial pathogen" in reference to a plant or seed modified in accordance with the invention means a transformed plant or seed in which growth of a given microbial pathogen is inhibited or prevented in a quantifiable amount over growth of a given microbial pathogen in a native, non-treated control plant or seed. Preferably, growth of a phytopathogenic microbe is suppressed by at least about 25% in comparison to microbial growth for a native control plant or seed. More preferably, microbial growth is suppressed by at least about 50%, and even more preferably by at least about 80%, and will depend upon various factors such as soil or storage conditions, climate, plant or seed type, and the like.

"Seed" is meant to encompass all seed components, including, for example, the coleoptile and leaves, radicle and coleorhiza, scutulum, starchy endosperm, aleurone layer, pericarp and/or testa, either during seed maturation or seed germination.

"Seed development" refers to any seed condition, from fertilization to late-stage germination, characterized by induction of one or more enzymes in the seed. Seed development refers to both seed maturation and seed germination.

"Secondary metabolite" is a metabolite that is present in one or more plant tissues, including seeds, but which is not essential for plant survival.

"Intermediate secondary metabolite" is a secondary metabolite that is further converted by plant enzymes to another secondary metabolite.

An intermediate secondary metabolite is present at "above-normal levels" if the metabolite is present in measurable amounts in transformed, but not in wildtype control plant seed tissue, or is present at levels of at least about 10%, preferably 25%, and more preferably at least 50% higher than measured in wildtype seed tissue of the same stage.

"Chimeric gene" as defined herein refers to a non-native gene (*i.e.*, one which has been introduced into a host) which is composed of parts of different genes, including genetic regulatory elements. A chimeric gene construct for plant/seed transformation is typically composed of a transcriptional regulatory region (promoter) operably linked to a heterologous protein coding sequence or to an inhibitory gene sequence, or in a selectable-marker chimeric gene, to a selectable marker gene encoding a protein conferring antibiotic resistance to transformed plant cells. A typical chimeric gene of the present invention, for transformation into a plant, may include a transcriptional regulatory region inducible during seed development (*e.g.*, a seed specific promoter such as a rice glutelin gene promoter, or a tissue or stage-specific promoter), the inhibitory sequence, and a terminator sequence.

By "promoter" or "transcriptional regulatory region" is meant a sequence of DNA that directs or regulates transcription of a downstream gene heterologous to the promoter, and includes promoters derived by means of ligation with operator regions, random or controlled mutagenesis, addition or duplication of enhancer sequences, addition or modification with synthetic linkers, and the like.

A "seed-induced promoter" or seed developmental stage specific promoter is one which is inducible in seeds during seed development, either under the control of endogenous factors present in the seed, plant hormones, such as abscissic or gibberellic acid, or physical stimuli, *e.g.*, heat and moisture. The seed-induced promoter may be "seed-specific", meaning it is induced preferentially in seeds relative to other tissue (Knutzon, *et al.*, 1992; Bustos, *et al.*, 1991; Lam and Chua, 1991; and Stayton, *et al.*, 1991), "seed-layer-specific", meaning it is induced preferentially in certain seed cells, *e.g.*, testa-layer cells, or "seed-stage-specific", meaning it is induced during certain stages of seed maturation or germination.

An "inhibitory gene sequence" is a gene sequence which (i) encodes an enzyme which is capable of increasing the levels of a selected intermediate secondary metabolite in seeds, or (ii) inhibits expression of an enzyme which converts a selected intermediate secondary metabolite to another, typically less fungi-toxic, secondary metabolite.

The mRNA transcript produced by a chimeric gene during seed maturation or seed germination is referred to as an "inhibitory transcript RNA", meaning the transcript RNA has the "biological activity" of (i) blocking the cellular expression of an enzyme that converts the selected metabolite to another metabolite less toxic to the pathogen, or (ii) being translated to an enzyme that is effective to cause an increase of the level of a selected intermediate secondary to above-normal levels in a seed.

The nucleotide sequences described herein, particularly inhibitory gene sequences, are meant to encompass variants possessing essentially the same "sequence identity". A gene of the same function, considered to share sequence identity with a subject sequence, such as an inhibitory gene sequence or a particular region or regions thereof, has at least about 65%, preferably 70%, more preferably from about 70-75%, or even more preferably from about 75 to 80% or greater global sequence identity over a length of the subject sequence (*e.g.*, SEQ ID NOs:1-4). Regions or fragments forming a basis for determination of percent sequence identity are typically at least 15 nucleotides in length, preferably at least 18-20 nucleotides in length, and more preferably at least 25 nucleotides in length.

"Percent (%) nucleic acid sequence identity" is the percentage of nucleotides in a candidate sequence that are identical with the nucleotides in the native sequence or a subject sequence, after aligning the sequences and introducing gaps, if necessary, to achieve the maximum percent sequence identity. Alignment for purposes of determining percent nucleic acid sequence identity can be achieved in various ways that are within the skill in the art, for instance, using publicly available computer software such as ALIGN or Megalign (DNASTAR) software. Those skilled in the art can determine appropriate parameters for measuring alignment, including any algorithms needed to achieve maximal alignment over the length of the sequences being compared.

A nucleic acid fragment is considered to be substantially identical in sequence to another polynucleotide if it is capable of "selectively hybridizing" to such a polynucleotide. A nucleic acid fragment is considered to be selectively hybridizable to an subject polynucleotide (*e.g.*, SEQ ID NO:1) if it is capable of specifically hybridizing to the polynucleotide sequence, its complement, or a variant thereof, but not to unrelated, non-identical sequences, under high stringency (*i.e.*, selective or sequence specific hybridization) conditions. In reference to SEQ ID NO:1, such a selectively hybridizable sequence is considered a DHQR gene. High stringency conditions are known in the art; see for example Maniatis, *et al.*, MOLECULAR CLONING: A LABORATORY MANUAL, 2d Edition

(1989), and SHORT PROTOCOLS IN MOLECULAR BIOLOGY, Ausubel, *et al.* (Ed). Selective hybridization conditions are defined herein as hybridization at ~45°C in ~1.1M salt followed by at least one wash at 37°C in 0.3M salt.

In the context of the present invention, the phrase "nucleic acid sequences," when referring to sequences which encode a protein, polypeptide, or peptide, is meant to include degenerate nucleic acid sequences which encode identical protein, polypeptide or peptide sequences, protein variants, as well as the disclosed sequence.

"Percent (%) amino acid sequence identity" with respect to the protein and enzyme amino acid sequences identified herein is defined as the percentage of amino acid residues in a candidate sequence that are identical with the amino acid residues in the native sequence, after aligning the sequences and introducing gaps, if necessary, to achieve the maximum percent sequence identity, and not considering any conservative substitutions as part of the sequence identity.

"Identical" in this context means identical amino acids at corresponding positions in the two sequences which are being compared.

"Similarity" in this context includes amino acids which are identical and those which are similar (functionally equivalent), with identity being preferred. Similarity may be determined using standard techniques known in the art, such as the Best Fit sequence program described by Devereux, *et al.* (1984), preferably using the default settings, or the BLASTX program (Altshul, *et al.*, 1996). The alignment may include the introduction of gaps in the sequences to be aligned. In addition, for sequences which contain either more or fewer amino acids than the relevant native proteins, it is understood that the percentage of similarity will be determined based on the number of homologous amino acids in relation to the total number of amino acids. Alignment for purposes of determining percent amino acid sequence identity can be achieved in various ways that are within the skill in the art, for instance, using publicly available computer software such as LALIGN or Megalign (DNASTAR) software with default parameters). The LALIGN program is found in the FASTA version 1.7 suite of sequence comparison programs (Pearson, *et al.*, 1988; Pearson, 1990; program available from William R. Pearson, Department of Biological Chemistry, Box 440, Jordan Hall, Charlottesville, VA). Those skilled in the art can determine appropriate parameters for measuring alignment, including any algorithms needed to achieve maximal alignment over the sequences being compared.

A "protein or enzyme variant" means a protein or enzyme which comprises the biological activity of the native protein or enzyme and is further defined as having at least about 80% amino acid sequence identity with the "native protein or enzyme". Such a "protein or enzyme variant" includes, for example, an amino acid sequence for DHQR wherein one or more amino acid residues are added, or deleted, at the N-or C-terminus of the sequence, or substituted for one or more amino acid residues

within the se[redacted]e. Ordinarily, a "protein or enzyme varia[redacted]ill have at least about 80% amino acid sequence identity, preferably at least about 85% amino acid sequence identity, more preferably at least about 90% amino acid sequence identity and even more preferably at least about 95% amino acid sequence identity with the amino acid sequence of the native protein or enzyme.

A nucleic acid sequence is "operably linked" to another nucleic acid sequence when it is placed into a functional relationship with such a sequence. For example, DNA for a presequence or secretory leader is operably linked to DNA for a polypeptide if it is expressed as a preprotein that participates in the secretion of the polypeptide; a promoter or enhancer is operably linked to a coding sequence if it affects the transcription of the sequence; a ribosome binding site is operably linked to a coding sequence if it is positioned so as to facilitate translation. Generally, "operably linked" means that the DNA sequences being linked are contiguous, and, in the case of a secretory leader, contiguous and in reading phase. However, enhancers do not have to be contiguous. "Linking" (in the context of nucleic acids being operably linked) is accomplished by ligation at convenient restriction sites, and, if such sites do not exist, synthetic oligonucleotide adaptors or linkers are used in accordance with conventional practice.

"Stably transformed" as used herein refers to a plant cell or plant or seed that has foreign nucleic acid integrated into its genome which is maintained through at least two or more generations.

"DFR" and "DHQR" are synonymous, and refer to the enzyme dihydroflavanol reductase (DFR), also referred to as dihydroflavanol-4-reductase, also known as dihydroquercetin reductase (DHQR).

"DHQ" refers to dihydroquercetin, the structure of which is illustrated in Fig. 2B.

"DAA" is an abbreviation for days after anthesis.

II. Intermediate Secondary Metabolites

In practicing the method of the invention, there is first identified an intermediate secondary metabolite that can be shown to inhibit infection of a plant pathogen in monocot seeds, at above-normal levels of the metabolite.

A variety of classes of secondary metabolites may be investigated for their ability to inhibit phytopathogens at above-normal levels of the metabolite. Included here as suitable classes are the flavonoids, *e.g.*, flavonol quercetin, flavon-4-ols, luteoforol, apiforol, flavones, flavonones, dihydroquercetin (DHQ), dihydromyricetin, catechin, eriodictyol, procyanidin, leucocyanidin, and prodelphinidin; and cyclic hydroxamates such as benzoxazin. In corn, the cyclic hydroxamates protect against *Helminthosporium turcicum* and the European cornborer *Ostrinia nubilalis*. In the corn mutant

Bx 1, benzoxaz[redacted] not produced, and the mutant plants exhibit sensitivity to these pathogens (Couture, *et al.*, 1971).

Flavonoids, which represent an exemplary class of secondary metabolites for use in the invention, are defined generally as aromatic oxygen containing heterocyclic compounds that contribute to plant pigmentation and other physiological properties. Flavonoid pathways have been studied in many different plant species; the enzymatic pathway for biosynthesis of flavonoids in barley is presented as Figs. 2A-C.

The formation of the compounds indicated in Figs. 2A-C is dependent upon the activity of the initial enzyme chalcone synthase (CHS), which catalyses the production of chalcone from three molecules of malonyl-Coenzyme A and one molecule of coumaroyl- Coenzyme A. The flavones and flavonols are intermediates in the flavonoid pathway, being converted in the testa to the final products, procyanidin B-3 and prodelphinidin B-3, and in other parts of the plant to cyanidin-3-glucoside and anthocyanidins.

In barley, formation of proanthocyanidins and anthocyanins is controlled by the *Ant* genes (Jende-Strid, 1993, 1995), some of which are indicated in Fig. 2B. In some loci a considerable number of mutant alleles are available (Table I) (Jende-Strid, 1995). The enzymes involved in the formation of procyanidin B-3 (a dimer of (+)-catechin) and prodelphinidin B-3 (a dimer of (+)-gallocatechin) in barley are summarized in Table 1.

Table 1: Flavonoid Pathway of Barley

Enzyme	Product	Loci	Mutant Alleles	cDNA	Genomic Clone
Chalcone synthase	Naringenine chalcone	Several	-	+	+
Chalcone isomerase	Naringenine	*Ant*30	4	-	-
Flavonoid 3' hydroxylase	Eriodictyon	Several	-	-	-
Flavonoid 3',5' hydroxylase	Penta hydroxy flavanone	Several	-	-	-
Flavanone 3-hydroxylase	(+)-dihydroquercetin	*Ant*17	177	+	-
"	"	*Ant*22	6	-	-
Dihydroflavonol reductase	(+)-*cis*-leucocyanidin	*Ant*18	142	+	+
Leucocyanidin reductase	Catechin, gallocatechin	*Ant*19	1	-	-
"	"	*Ant*25	10	-	-
"	"	*Ant*28	9	-	-
"	"	*Ant*28	9	-	-
"	"	*Ant*29	3	-	-
Flavonol condensing enzyme	Procyanidin B-3 (-)	*Ant*26	8	-	-
"	Prodelphinidin B-3 (-)	*Ant*27	5	-	-

A. Secondary Metabolite Mutants

A large number of monocot mutants which are characterized by increases or decreases in certain selected secondary metabolites have been described. See generally, Kristiansen and Rohde (1991), Kristiansen (1984), and Meldgaard (1992), which describe methods for producing and characterizing monocot mutations characterized by above- or below-normal levels of a given metabolite.

Mutations that affect the levels of one or more secondary metabolites in a plant can include (i) inactivation of key enzymes in the secondary metabolite pathways, leading to the buildup of metabolites upstream of the mutation and reduction in metabolite levels downstream of the mutation (Meldgaard, 1992; Kristiansen and Rohde, 1991), and (ii) inactivation of a transcription factor responsible for the expression of one or more metabolites (Meldgaard, 1992; Byrne, 1996). Other mechanisms, such as loss of a protein involved in regulating levels of a key secondary metabolite, are possible.

By way of example, a number of barley mutants that affect flavonoid levels have been produced (Jende-Strid, 1993, 1995). *Ant*18 is the *Hordeum vulgare* (barley) structural gene encoding dihydroflavonol reductase (DFR), also referred to as dihydroquercetin reductase (DHQR), (Kristiansen and Rohde, 1991; Olsen, *et al.*, 1993). As illustrated in Fig. 2B, the single copy *Ant*18 gene encodes DHQR, which catalyzes the last common step in the flavonoid biosynthetic pathway leading to anthocyanins and proanthocyanidins.

Structural genes encoding DHQR have been cloned in other plant species including *Z. mays, Antirrhinum majus, and P. hybrida* (Kristiansen and Rohde, 1991). In an NADPH-dependent reaction, this enzyme reduces dihydroflavonols to 3,4-*cis*-leucoanthocyanidins as indicated in Fig. 5. The structure of the DHQR gene (*Ant*18 gene) is discussed further below.

In experiments performed in support of the invention, a number of barley mutants blocked in the synthesis of flavonoids in the testa cells of the grain were utilized to identify secondary metabolites capable of preventing or inhibiting *Fusarium* infection in monocot seeds.

The preparation of exemplary *ant*18 mutants, *ant*18-159, *ant*18-161, *ant*-18-162, and *ant*18-164, is described in Example 1. Barley mutants are typically prepared by treatment with sodium azide, due to the fact that the mutagen can be readily applied to dry and presoaked grains, and induces few, if any, chromosome aberrations (Olsen, *et al.*, 1993). Following mutagenesis, growth and selection of the homozygous *ant*18 mutants, the sequence of the mutant *ant*18 genes can be determined using standard recombinant techniques as described in detail in Example 1.

The base substitutions of representative mutant *ant*18 genes is provided in Example 1D. Mutant plants containing these *ant*18 alleles lack DHQR activity. For the mutant alleles *ant*18-159, *ant*18-162 and *ant*18-164, the levels of DHQR-specific transcripts are similar to wild-type plants

(Kristiansen and Rohde, 1991). Messenger RNA for DHQR is not detectable in *ant*18-164 cells, and has been attributed to mutations in the pre-mRNA splice donor site (Kristiansen and Rohde, 1991).

B. Pathogen-Resistant Mutants

Plant cells or plant tissue, and typically seeds, from mutated plants are tested for the ability to resist infection by a selected microbial pathogen, *e.g.*, a fungal pathogen such as *Alternaria, Fusarium, Helminthosporium*, *F. moniliforme*, *Cladosporium* or *Trichoderma* ssp.

Plant tissue, *e.g.*, seeds, identified as inhibiting growth of the pathogen are then further analyzed to identify (i) an intermediate secondary metabolite present in the seeds at elevated levels, and (ii) the phenotypic change in the plant cell that caused the increase in levels of the secondary metabolite, toxic to the pathogen.

By way of example, studies conducted in support of the present invention have shown that certain mutations in the *Ant*18 gene (i) were effective to provide an accumulation of dihydroquercetin in the testa/pericarp, and (ii) resulted in the formation of seeds that were extremely resistant to attack by *Fusarium*. In particular, *ant*18 barley mutants blocked in the synthesis of DHQR were found to specifically inhibit the growth and development of the fungus *Fusarium* (Example 2 and Table 2).

Table 2 provides a summary of representative wild type barley lines and mutants, their production of anthocyanin or proanthocyanidins, accumulation of particular flavonoids, and resistance to infection by *Fusarium*.

As can be seen from the results in Table 2, with the exception of the *ant*18 mutant, each of the exemplary *ant* mutants were more susceptible to infection by *Fusarium* than the corresponding wild type grains (Triumph, Alf, Grit). In contrast, the *ant*18-159 kernels exhibited superior resistance to infection to *Fusarium*, as shown in Fig. 3D.

After 5-6 days of incubation, the CZID agar on which *ant*13-152 and *ant*17-148 grains were positioned, turned dark red due to the aurofusarin pigment secreted by the fungus. In contrast, this color formation was observable only after 5-6 weeks in the agar under the *ant*18-159 grains. Kernels of *ant*18-159 showed consistently more resistance to *Fusarium* attack than any of the wild types tested.

The length of time it took for *ant*18-159 kernels to become overgrown with *F. culmorum* mycelium in comparison with *ant*13-152 kernels was also investigated. While *ant*13-152 was strongly infected and overgrown with mycelia following a one week incubation period, 6 weeks were necessary for *ant*18-159 to be overgrown to the same extent. Repetitions of infection experiments with *F. poae*, *F. graminearum* or *F. culmorum* at different spore-concentrations, supported these findings.

As previously described, null mutants in the *ant*18 gene encoding dihydroflavonol reductase accumulate dihydroquercetin. While the wild types accumulate about 130 mg catechin and

proanthocyani[redacted]downstream products) per 100 g dry weight, [redacted]e *ant*18 mutants (*e.g.*, *ant*18-102, -141, -159, -186) contain about 0.60 to 0.70 mg dihydroquercetin per 100 g dry weight (Kristiansen, 1984; Jende-Strid, 1985). However, this small amount of dihydroquercetin accumulated in the testa of *ant*18 mutants is effective to provide an extraordinary resistance to *Fusarium* infection (Figs. 3A-D), leading to the discovery of the present invention.

As further support for this finding, authentic dihydroquercetin inhibited growth on medium and more importantly, prevented macrospore formation (Example 6). *In planta*, dihydroquercetin prevented the penetration of the testa layer. Finally, the bioautographic assay on TLCs (Example 9) confirmed one aspect of the present invention, namely, that the *in-vivo* accumulation of dihydroquercetin in the outer seed layers imparts *Fusarium* resistance in the mutant.

III. Chimeric Genes for Plant Pathogen Resistance

Following the identification of a secondary intermediate metabolite effective to inhibit, at above-normal levels, infection by a given plant pathogen, a monocot plant is stably transformed with a chimeric gene construct having (i) a promoter (transcriptional regulatory region) induced during seed development, and (ii) an inhibitory gene sequence operably linked to the promoter. In one aspect, the chimeric gene is effective, during seed development and/or germination, to induce an inhibitory transcript RNA whose action at the transcript levels induced, leads to an accumulation of the secondary intermediate metabolite at levels toxic to the microbial pathogen.

Additional structural genes of interest may also be employed in the present construct, either native, mutant native, or foreign to the plant host, and may be provided in a sense or antisense orientation. For native and mutant genes, the gene may, for example, provide an improved nutrient source, herbicide resistance, etc., while foreign genes may provide enhancement of native capabilities or production of foreign products as a result of expression of one or more foreign genes. Typically, such a structural sequence will be under the control of a seed-inducible promoter of the type described herein. Resultant seeds and seed-derived products (*e.g.*, seed components, seed extracts, malt and its derivatives, recombinant proteins, and the like) will thereby exhibit an increased resistance to contamination by microbial pathogens, resulting in high purity seed products.

A. Seed Promoters

The transcription regulatory or promoter region of the chimeric gene is preferably a seed-induced promoter, and more preferably, a testa-specific promoter such as a leucocyanidin reductase gene promoter or a promoter from the newly identified testa-specific gene described in Example 8 (provisionally designated as "DP3"), capable of directing expression of a gene product under its control to a specific plant tissue or cell type, such as the testa layer. Promoter sequences for

regulating transcription of an inhibitory gene sequence include naturally-occurring promoters, or regions thereof capable of directing seed-specific transcription, and hybrid promoters, which combine elements of more than one promoter and are well known in the art.

Preferably, the promoter is derived from the same plant species as the plant in which the chimeric gene construct is to be introduced; promoters for use in the invention are typically derived from cereals such as rice, barley, wheat, oat, rye, millet, triticale, sorghum, and corn. Alternatively, a seed-specific promoter from a non-cereal monocot may be used and modified according to known techniques for optimum seed-specific expression in a particular plant cell host. Numerous types of appropriate expression vectors, and suitable regulatory sequences are known in the art for a variety of plant host cells. In general, the transcriptional and translational regulatory sequences may include, but are not limited to, promoter sequences, ribosomal binding sites, transcriptional start and stop sequences, translational start and stop sequences, and enhancer or activator sequences.

Identifying suitable seed-inducible or seed-regulated transcriptional initiation regions may be achieved in a number of ways, such as those described in U.S. Patent No. 5,753,475, which is incorporated herein by reference in its entirety. Promoters may be isolated from various seed tissues and/or at various stages of seed development by a variety of techniques routinely used by those of skill in the art. Such techniques include, but are not limited to: (1) use of conventional hybridization techniques and known promoters from a different species, tissue and/or developmental stage to obtain related sequences from the target monocot species, seed tissue or seed developmental stage, (2) subtractive hybridization (Lee, *et al.*, 1991), (3) differential display (Liang and Pardee, 1992; Bauer, *et al.*, 1993), and (4) selective amplification via biotin- and restriction-mediated enrichment, SABRE (Lavery et al., 1997).

By way of example of one approach for obtaining a seed-inducible promoter, a seed protein is at least partially sequenced, for designing a probe to identify messenger RNA specific to seed. To further enhance the concentration of seed-associated mRNA, cDNA is prepared and the cDNA subtracted with messenger RNA or cDNA from non-seed associated cells. The residual cDNA is then used to probe the genome for complementary sequences using an appropriate library prepared from plant cells. Sequences which hybridize to the cDNA are then isolated, manipulated, and the 5' untranslated region associated with the coding region is isolated and used in expression constructs to verify the transcriptional (*i.e.*, seed tissue or stage specific) activity of the 5'-untranslated region.

In employing a subtractive hybridization approach, sequences present in one nucleic acid sample type and not in another are determined by subtractive hybridization. Using this protocol, nucleic acid extracts are prepared from two different samples, hybridized and sequences that do not hybridize are then amplified (Lee, *et al.*, 1991).

To determine the sequence of a known promoter from a different monocot type, conventional hybridization techniques are typically applied. For example, to identify a wheat endosperm-specific promoter, a cDNA library from wheat seeds, or wheat endosperm, is amplified using probes containing consensus sequences from, *e.g.* a known corn promoter sequence (the maize zein promoter) or more preferably, its associated structural gene sequence. Amplified wheat-cDNA fragments can then be confirmed as corresponding to an endosperm-specific promoter, *e.g.*, by Southern blotting with radio-labeled probes from the zein or other known endosperm-specific promoter sequences.

Promoters from seed tissue specific genes such as those described in Müller and Knudsen (1993) and in the references contained therein, the contents of which are expressly incorporated herein by reference, *e.g.*, the barley C hordein gene (GenBank Accession No. X60037), are suitable for use in the chimeric gene constructs described herein.

Specifically, representative seed-induced promoters for use in the invention include the promoters from the rice glutelin multigene family, Gt1, Gt2, Gt3, GluA-3, and GluB-1. Promoter regions for these genes are described, for example, in Takaiwa, *et al.*, 1987 (rice glutelin gene, GenBank Accession Nos. D26365 and D26364), Takaiwa, *et al.*, 1991 (rice GluA-3 gene, GenBank Accession No. X54313), Takaiwa, *et al.*, 1991a (rice glutelin gene, GenBank Accession No. Y00687), Takaiwa, *et al*, 1991b (rice Glu-B gene, GenBank Accession No. X54193; rice GluB-2 gene, GenBank Accession No. X54192; rice GluB-1 gene, Genbank Accession No. X54314); Okita, *et al.*, 1989 (rice Gt2 gene, GenBank Accession No. L36819 M28157; rice Gt3 gene, GenBank Accession No. M28158; rice Gt1 gene, GenBank Accession No. M28156; rice glutelin gene, GenBank Accession Nos. D26363, D26366, D26367, D26368 and D26369); Abe, *et al.*, 1989 (rice prepro-glutelin gene, GenBank Accession No. D00584); Kim and Wu, 1990 (rice glutelin gene, GenBank Accession No. X52153). These promoters are active during seed development and direct endosperm-specific expression (Takaiwa, *et al.*, 1991a, 1991b; Okita, *et al.*, 1989; Abe, *et al.*, 1989; Kim and Wu, 1990).

Other suitable seed-induced promoters include the promoter region from the rice prolamine gene, GenBank Accession No. D73384 (Nakase, *et al.*, 1996).

Additional representative seed-induced promoters include the barley B22EL8 gene promoter, which directs expression in immature aleurone layers (Klemsdal, *et al.*, 1991); the promoter for the barley LTp gene (GenBank Accession No. X57270); the barley β-amylase (Kreis, *et al.*, 1987; GenBank Accession No. X52321 M36599) and β-glucanase gene promoters (Wolf, 1992) *e.g.*, the barley G1b gene promoter, GenBank Accession No. X56775, the barley CMd gene promoter (Halford, *et al.*, 1988; GenBank Accession No. X13198), and promoters from the barley hordein gene family of seed storage proteins, *e.g.*, B-, C-, and D-hordein genes (Sorensen, *et al.*, 1996 and references therein; Sorensen, 1992; Brandt, *et al.*, 1985 (hordein B1 gene promoter, GenBank

Accession No. X87232); Entwistle, 1988 (barley hordein C promoter, GenBank Accession No. M36941), Entwistle, *et al.*, 1991; Müller and Knudsen, 1993, barley hor1-17 gene, GenBank Accession No. X60037). Hordein gene promoters such as the Hor3 gene promoter (Sorensen, 1996; GenBank Accession No. X84368) direct the specific expression of the corresponding genes in the endosperm. The endosperm-specific expression regulated by both the barley B-hordein promoter and the maize zein promoter (GenBank Accession No. X63667 S39626 S39890) has been correlated with hypomethylation of the corresponding structural genes (Sorensen, *et al.*, 1996; Bianchi and Viotti, 1988).

Additional seed-induced promoters for use in the invention are the maize zein gene promoter (Bianchi and Viotti, 1988), and promoters from wheat glutenin genes, such as those described in Anderson, *et al.*, 1989 and Halford, *et al.*, 1989. Representative wheat glutenin gene sequences as sources for suitable promoters include GenBank Accession Nos. U86028, U86029, and U86030.

Also for use in the present invention are seed induced corn promoters. Promoters from cloned seed tissue specifically-expressed genes in corn include: the corn *O2*-opaque 2 gene promoter (Schmidt, *et al.*, 1987), GenBank Accession No. M29411; the corn *Sh2*-shrunken 2 gene promoter (Shaw and Hannah, 1992), GenBank Accession No. S48563; the *Bt2*-brittle 2 gene promoter (Bae, *et al.*, 1990); and the *Zp1* zein gene promoter (Burr, *et al.*, 1982). These promoters induce endosperm-specific expression, while the *Agp1* and *Agp2* gene promoters (Giroux and Hannah, 1994) are embryo-specific promoters. The sequences of the above-described promoters, and/or the structural sequences from which such promoters may obtained, are herein incorporated by reference.

Any of the above promoters may also be obtained from an alternative monocot species. For example, a promoter such as the Gt1 gene promoter from rice may be isolated from other cereal-derived nucleic acid containing extracts, *e.g.*, wheat, oat, or the like, using conventional hybridization techniques known in the art.

The identification and/or isolation of a testa-specific promoter for directing expression in the testa layer of developing seeds will now be described.

B. Testa-Specific Promoters

The testa, or seed coat, is an outer layer of cells adjacent to the aleurone layer surrounding the endosperm. The testa is composed of an inner and outer cuticle, often impregnated with waxes and fats, and one or more layers of thick-walled protective cells. The testa may contain layers of crystal containing cells composed of calcium oxalate or carbonate; the seed coat may also contain mucilaginous cells that burst upon contact with water, to provide a water-retaining barrier. The testa layer in seeds functions to provide a protective barrier between the embryo and the external environment.

To isolate a testa-specific promoter, *i.e.*, a promoter that is selectively induced in the testa/pericarp layer of seeds, cDNA difference techniques can be employed to identify a gene that is expressed in testa layer cells, but not in other seed cells, *e.g.*, aleurone cells. One exemplary difference technique, representational difference analysis, is the one detailed in Example 8, and illustrated in Fig. 6. Briefly, cDNA's from a target tissue (testa cells) and driver tissue (aleurone) were modified to have end linkers, and amplified by PCR using primers complementary to the linker sequences. As shown in Fig. 6, the amplified tester and driver sequences are end modified so that the tester sequences have end linkers and the driver sequences do not. Excess driver fragments are added to the tester sequences and the two fragment groups hybridized, then amplified by PCR, using primers specific for the tester end linkers. Only unique tester sequences, *i.e.*, cDNA sequences present in testa but not aleurone cells, are exponentially amplified, as indicated in the figure.

The first difference product (DP1) shows two major bands. After two additional difference amplification steps, as above, the material purified to two clearly identified bands. These bands, identified as RDA-1 (SEQ ID NO:6) and RDA-2 (SEQ ID NO:7), have the sequences shown in Figs. 7A and 7B, respectively. These sequences likely correspond to regions of a testa-specific gene such as *Ant*13.

Since the *ant*13 barley mutant contains a mutation in the *Ant*13 barley gene encoding the transcription factor required to express all genes of the anthocyanidin and proanthocyanidin pathway, including the structural gene for leucocyanidin reductase, which is expressed only in testa cells, it was of interest to determine whether the identified gene was expressed in the testa layer of wildtype, but not *ant*13 barley mutants. This was done by performing PCR amplification of cDNA fragments obtained from the testa layer of wildtype and *ant*13 mutants, using primers constructed from the above RDA-1 or RDA-2 sequences, as discussed in Example 8 with respect to Fig. 8. A Southern blot analysis of amplicons obtained with the primers derived from RDA-1 indicate that this sequence is only present among mRNA from wild type testa tissue, but absent in the mutant testa cells and wild type leaf tissue. Similarly, an analysis of RDA-2 reveals that this sequence is likewise only present among mRNA from wild type testa tissue. These results demonstrate that the identified testa-specific gene, herein provisionally designated as "DP3" is not expressed in the testa layer of *ant*13 mutants, suggesting that the gene is involved in the anthocyanidin and proanthocyanidin pathway. Further, the gene is not expressed in leaf tissue.

A preferred method for obtaining testa-specific regulatory sequences is selective amplification via biotin- and restriction-mediated enrichment, SABRE (Lavery, *et al.*, 1997), as discussed above. In this technique, species more abundant in one double-stranded DNA population (the tester, *e.g.*, testa cell cDNA) than another (the driver, *e.g.* aleurone cDNA) are enriched relative to species equally expressed in both populations. Briefly, a mixture of both populations is denatured and allowed to

reassociate. Only double-stranded molecules in which both strands are derived from the tester population (tester homohybrids) are then isolated. This population is enriched for species with greater abundance in the tester relative to the driver because more complementary DNA strands are contributed by the tester population than the driver population relative to those of species equally expressed in both populations.

Tester homohybrids are then specifically purified by PCR amplification to generate driver and tester populations using primers that are nearly identical, but have two significant differences: the tester PCR primer is biotinylated at the 5' end and contains the recognition site for the restriction enzyme EcoRI near its 5' end. In contrast, the driver PCR primer is non-biotinylated and contains a mutation of the EcoRI site. After extensive hybridization of the driver and tester populations with driver DNA in excess, the hybrids are digested to remove single-stranded molecules. Biotin-containing tester homohybrids and driver-tester heterohybrids are captured on streptavidin-coated magnetic beads. The bead-DNA complexes are then incubated with EcoRI, which recognizes and cuts the double stranded palindrome present in the tester homohybrid primer sequences releasing them from the beads. The driver-tester heterohybrids are not cleaved by the enzyme, and tester homohybrids are can then be amplified by PCR for another round of selection or for isolation and subcloning of enriched species.

Once a testa-specific gene is isolated, for example by the difference or SABRE techniques discussed above, the full gene is sequenced and the promoter region is identified. Identifying the promoter region is performed by standard analysis, for example, by identifying a start codon at the beginning of an open reading frame, and promoter-specific sequences, *e.g.*, a TATA box and transcription initiation site upstream of the start codon. The promoter can then be isolated and cloned by standard recombinant methods, for use in the chimeric gene of the invention.

Following isolation of the promoter, expression specificity can be further confirmed by fusing the promoter in-frame to a reporter such as the synthetic gene of the jelly fish green fluorescence protein, engineered for high level expression in higher plants (Chiu, *et al.*, 1996). The promoter-reporter gene construct is then delivered on gold particles by the above-described biolistic method into the developing testa and control tissues (*e.g.*, leaf, stem). Expression specificity can also be confirmed by Northern blot analysis using mRNA preparations from a variety of barley tissues.

C. Antisense Inhibitory Sequences

In one general embodiment, the inhibitory transcript RNA produces its inhibitory biological effect by inhibiting expression of an enzyme that functions to convert the toxic secondary metabolite to another, typically less toxic, metabolite.

The enzyme whose expression is to be inhibited can be identified, in many cases, from known metabolic pathway reactions, such as those illustrated for flavonoid compounds in Fig. 2. By way of example, and with reference to this figure, dihydroquercetin is converted to cis-3,4-leucoanthocyanidin by DHQR. Since buildup of dihydroquercetin by lack of enzymatically active DHQR or defective DHQR transcript maturation (Olsen, *et al.*, 1993) has been shown herein to confer seed resistance to *Fusarium* infection, antisense inhibition of the gene encoding DHQR in testa cells will lead to the accumulation of dihydroquercetin. Antisense inhibition with an antisense gene or part of such a gene can take place in two independent ways: the antisense gene can control the transcript level in the nucleus and/or the translation efficiency of the target mRNA in the cytoplasm (Cornelissen and Vandewiele, 1989; Cornelissen, 1989).

To inhibit expression of DHQR, a chimeric gene is constructed which contains a seed tissue specific promoter recognized by the plant cell host, fused in-frame to the inverted gene coding for DHQR and a transcription terminator sequence. The sense strand of the inhibitory gene sequence is at least partially complementary to the transcribed region of the sense strand of the DHQR gene. That is, the orientation of the inhibitory gene sequence in the chimeric gene is in the reverse direction (3'-5'), so that RNA transcribed from the plasmid is complementary in sequence to the mRNA transcribed from the corresponding endogenous DHQR gene. This RNA will either limit the transcript level in the nucleus and/or diminish, respectively eliminate, the translation of the endogenous DHQR mRNA.

An inhibitory gene sequence for use in the invention will be at least partially complementary to an endogenous DNA sequence transcribed by the host. That is to say, an inhibitory sequence complementary to a sequence of the messenger RNA will usually be at least 10-20 nucleotides in length, more typically at least about 30 nucleotides in length, preferably at least about 50 nucleotides, and more preferably 100 nucleotides or more, usually being fewer than about 5000 nucleotides.

As described above, the structure of the *Ant*18 gene which encodes the DHQR enzyme in barley is known and is presented herein as SEQ ID NO:1 (GenBank Accession No. S69616). It will be understood that nucleotide differences in the *Ant*18 gene may exist among different barley genotypes and among various monocots, and that the invention is intended to encompass such variants. It will be further understood that part of the antisense gene, *i.e.*, comprising nucleotide sequences as short as 10-20 bases, can also be used for antisense inhibition.

Construction of an exemplary chimeric gene containing a DHQR inhibitory sequence, *e.g.*, an antisense DHQR gene, will now be described.

As an exemplary first step in constructing such a chimeric gene containing an antisense DHQR construct, the dihydroquecetin reductase coding sequence (SEQ ID NO:1) is used as a template. The ATG start codon is located 57 bases downstream of the transcriptional start site, at position +56. A promoter such as those described in B.1., *e.g.*, a testa-specific "DP3" gene promoter, is used to direct

transcription of the anti-sense dihydroquecetin reductase gene in the testa layer of transgenic barley seeds.

Construction of an exemplary anti-sense DHQR gene is presented schematically in Fig. 9 (ant18-AS). As illustrated therein, a complementary DNA clone (*Ant*18-COMP) of the *Ant*18 DHQR gene with an introduced *Nco*I site as the initiation codon can be used as the donor dihydroquercetin reductase coding sequence (Fig. 9A). Alternatively, the N-terminal (5') portion of the gene may be used, and the two *Nco*I sites utilized to move the N-terminal portion of the gene into the *Hinc*II site of mp7, thereby eliminating the need for a partial *Nco*I digest.

A cDNA fragment from a DHQR clone for constructing *Ant*18-COMP can be obtained essentially as follows. A lambda cDNA library is prepared as described in Kristiansen and Rohde (1991) and screened with barley *Ant*18 DNA. Positive clones are identified, and a cDNA insert about 20 nucleotides to 1.3kb in length, corresponding to a portion of the *Ant*18 gene sequence, is subcloned into a subcloning vector such as pUC9.

Returning now to Fig. 9, the *Ant*18-COMP mutant (cDNA clone) is partially digested with a restriction enzyme, *e.g.*, *Nco*I, and completely with *Eco*RI, and the sticky ends are made blunt using the Klenow fragment of DNA polymerase I and dNTPs (Fig. 9B). The resulting blunt ended fragment is then subcloned, *e.g.*, into the *Hind*II site of phage M13 mp7 (Fig. 9C), to allow isolation of a *Bam*HI fragment (Fig. 9D). This fragment can then be cloned into the *Bam*HI site separating the promoter and a nopaline synthase 3' flanking region containing a polyA tail (Fig. 9E). A clone containing the DHQR coding sequence in reverse orientation behind the promoter is then used to isolate the entire gene fragment, *e.g.*, *Eco*RI-*Hind*III fragment, which can then be cloned into the polylinker site of a suitable plant transformation vector, to form *Ant*18-AS.

Additional DHQR inhibitory sequences for use in chimeric gene constructs for preventing transcription and/or translation of the *Ant*18 gene are *ant*18 gene mutants of the type described herein (*e.g.*, Example 1, section IIB).

Referring to the sequence presented as SEQ ID NO:1, the start of transcription is at the +1 nucleotide (C) while the translation start codon is located 57 bp downstream of the start of transcription. A putative TATA box is located upstream of the transcription start at position -24 to -31. A CCAACCT sequence, resembling the CAAT box consensus is located at position -136 to -142. In looking at the 3' region, the first stop codon is a TAA at position 1432, followed in-frame by a second TAA codon. A single polyadenylation signal, AATAAA is located 138 based downstream of the first TAA translation stop codon and 13 bp before the 3' end of the isolated cDNA.

Similar strategies (Reddy, *et al.*) are applied to identifying the sequence of DHQR genes from monocots other than corn and rice, as described above. By way of example, to identify the wheat DHQR gene, a cDNA library from wheat seeds, or wheat testa layer cells, is amplified using probes

containing consensus sequences from the known rice (SEQ ID NO:3; GenBank Accession No. Y07956), barley (SEQ ID NO:1; GenBank Accession No. S69616), corn (SEQ ID NO:2; zea mays A1 gene, GenBank Accession No. X05068), alfalfa (SEQ ID NO:5, GenBank Accession No. X80222) and sorghum DHQR (SEQ ID NO:4, GenBank Accession No. U87454) genes. The sequences and locations of the genes identified by SEQ ID NOs: 2-4 are conserved in these three cereals, including the neighboring *Sh2* gene (Chen, *et al.*, 1997).

Amplified wheat-cDNA fragments can then be confirmed as belonging to the DHQR gene by Southern blotting with radiolabeled probes from one of the known DHQR genes.

The identified cDNA is then sequenced, determined to correspond to full-length cDNA, and cloned in a suitable vector, for use in chimeric gene construction according to the methods described herein. From this sequence, the antisense gene, corresponding to the full cDNA length gene or the N-terminal portion thereof, is constructed. Thus an antisense sequence complementary to this sequence, would be an effective inhibitory sequence against barley, wheat, rice, corn, and other cereal transcripts. Construction of the corresponding antisense genes for wheat, corn, rice, and related monocots is carried out essentially as described above for barley, and as described generally in U.S. Patent No. 5,759,829, the contents of which is expressly incorporated herein by reference in its entirety.

It will be appreciated that the inhibitory transcript RNA may also be designed to block gene transcription by binding to one strand of the gene during transcription. The transcript in this case may have either sense or antisense orientation with respect to the target gene sequence. Further, the transcript can target any sequence of the gene, including transcription binding site(s) and intron regions, effective to block transcription of a full-length mRNA.

These non-cDNA sequences can be identified by isolation and sequencing of the DHQR gene from a genomic library, according to standard procedures.

D. Overexpression of Secondary Metabolite Genes

Alternatively, the transcript RNA produced by the inhibitory sequence may produce its inhibitory action by overexpressing an enzyme that is upstream of the toxic secondary metabolite, and which therefore can lead to an overproduction of the toxic metabolite. In the case of dihydroquercetin buildup as a mechanism of *Fusarium* resistance, any of the enzymes upstream of the DHQR enzyme, *e.g.*, chalcone synthase (van der Krol, *et al.*, 1988), may be selected for overexpression. Typically, the desired enzyme is one which can be identified as being rate limiting in the production of a selected secondary metabolite, *e.g.*, DHQ.

As an example, and with reference to Fig. 2, it has been observed that blocking enzymatic pathway 8 in the figure (the hydroxylase enzyme which catalyzes the conversion of eriodictyol to

DHQ) results in elevated levels of chrysoeriol, indicating that this reaction, *i.e.*, pathway 8, is a bottleneck or rate limiting step in the production of DHQ. Therefore, overexpressing this enzyme would be expected to result in elevated levels of DHQ.

The sequences of the structural genes for a variety of the enzymes upstream of dihydroquercetin are known. The promoter sequence in the chimeric gene is preferably one which is highly induced during seed development, such as those described in sections IIA. and IIB.

In yet another approach, inhibitory action is achieved by introducing a transgene coding for an enzyme that converts a toxic secondary metabolite to a non-toxic metabolite, *e.g.*, a DHQR gene, and which therefore can lead to an accumulation of the toxic secondary metabolite via co-suppression or silencing of both the endogeneous (host) and exogeneous (inserted) genes for the enzyme (Knowles, *et al.*, 1998; Hart, *et al.*, 1992; Cluster, *et al.*, 1996). Typically, the transgene or transcriptional cassette is composed of, in the 5' to 3' direction, a high level constitutive promoter, such as the Cauliflower Mosaic Virus (CaMV) 35 S promoter, operably linked to a sequence encoding an enzyme, which in the native plant host, converts a selected intermediate secondary metabolite to another, less fungi-toxic, secondary metabolite, *e.g.*, a DHQR gene (*e.g.*, SEQ ID NOs:1-3,5).

The construct is then introduced into a plant host as described herein, and depending upon the manner of introduction into the plant, other DNA sequences apparent to one skilled in the art may be required. Transformants are then analyzed to determine the extent of silencing of both host and transgene enzymatic activity. Transformants in which gene silencing is observed, *i.e.*, those transformants in which enzymatic activity is reduced by at least about 25%, typically by at least about 50%, and preferably by at least about 70% in comparison to a wild-type control plant, are then collected. Co-suppression plant transformants producing seeds which exhibit (i) a build-up of intermediate secondary metabolite, and (ii) enhanced resistance to a microbial pathogen are then identified, and optionally propagated.

E. Transcription and Translation Terminators

The expression cassette or chimeric gene in the transformation vector will typically have a transcriptional termination region at the opposite end from the transcription initiation regulatory region. The transcriptional termination region may normally be associated with the transcriptional initiation region or from a different gene. The transcriptional termination region may be selected, particularly for stability of the mRNA to enhance expression. Illustrative transcriptional termination regions include the NOS terminator from *Agrobacterium* Ti plasmid and the rice α-amylase terminator.

Polyadenylation tails, (Alber and Kawasaki, 1982) are also commonly added to the expression cassette to optimize high levels of transcription and proper transcription termination, respectively.

Polyadenylation sequences include, but are not limited to, the *Agrobacterium* octopine synthetase signal, Gielen, *et al.* (1984) or the nopaline synthase of the same species Depicker, *et al.* (1982).

Since the ultimate expression of the inhibitory sequence will be in a eukaryotic cell (in this case, a member of the grass family), it is desirable to determine whether any portion of the cloned gene contains sequences which will be processed out as introns by the host's splicing machinery. If so, site-directed mutagenesis of the "intron" region may be conducted to prevent losing a portion of the genetic message as a false intron code (Reed and Maniatis, 1985).

F. Construction of a Transformation Vector

Vectors containing a chimeric gene of the present invention are typically provided with selectable markers for use in plant cells, either on the same plasmid or in the form of separate plasmids, such as the *npt*II kanamycin resistance gene, for selection in kanamycin-containing media, or the phosphinothricin acetyltransferase gene, for selection in medium containing phosphinothricin (PPT).

The vectors may also include sequences that allow their selection and propagation in a secondary host, such as, sequences containing an origin of replication and a selectable marker such as antibiotic or herbicide resistance genes, *e.g.*, *hpt* (Hagio, *et al.*, 1995; and van der Elzer, 1985). Typical secondary hosts include bacteria and yeast. In a representative approach for forming an expression vector for use in the present invention, the secondary host is *Escherichia coli*, the origin of replication is a colE1-type, and the selectable marker is a gene encoding ampicillin resistance. Such sequences are well known in the art and are commercially available as well (*e.g.*, Clontech, Palo Alto, CA; Stratagene, La Jolla, CA).

A vector for use in the present invention may also be modified to form an intermediate plant transformation plasmid that contains a region of homology to an *Agrobacterium tumefaciens* vector, a T-DNA border region from *Agrobacterium tumefaciens*, and chimeric genes or expression cassettes (described above). Further, the vector may comprise a disarmed plant tumor inducing plasmid of *Agrobacterium tumefaciens*.

As described above, Fig. 9 shows steps in constructing an exemplary transformation vector for transforming barley plant cells capable of inhibiting transcription and/or translation of a DHQR gene, where the vector in Fig. 9 contains an antisense DHQR gene.

The vectors described above are suitable for use in a method of producing resistance to fusarium in seeds of a plant, where formation of an enzyme that metabolizes dihydroquercetin, *i.e.*, DHQR, is inhibited, to thereby provide an accumulation of dihydroquercetin in seeds of the transgenic plant. Promoters may be selected as indicated above. For example, for repressing DHQR expression in the seed coat layer of developing seeds, an antisense DHQR coding sequence may be placed under

the control of a testa specific promoter such as the leucocyanidin reductase gene promoter or a "DP3" gene promoter. Examples of other suitable promoters active during seed development, are the barley B22E gene promoter, the barley β-amylase and β-glucanase gene promoters, promoters from the barley hordein gene family, *e.g.*, B-, C-,D-, and those promoters discussed in section III.A.

IV. Stably Transformed Monocot Plants

Plant cells or tissues such as seeds derived from the members of the family are transformed with expression constructs (*i.e.*, plasmid DNA into which the gene of interest has been inserted) using a variety of standard techniques (*e.g.*, electroporation, protoplast fusion or microparticle bombardment). The expression construct includes a transcription regulatory region (promoter) whose activity is specifically inducible during seed development (a stage-specific promoter), or in particular plant tissues, *e.g.*, maturing seeds. In the present invention, particle bombardment is the preferred transformation procedure.

The construct also includes an inhibitory gene sequence whose transcription product is an polynucleotide effective to prevent (i) transcription of the gene encoding DHQR (ant18), or (ii) translation of the DHQR mRNA. Preferably, the inhibitory sequence is an anti-sense DHQR gene effective to allow an accumulation of dihydroquercetin within transformed cells. Preferably, the inhibitory sequence gene is placed under the control of a seed-induced promoter. In one embodiment, the promoter is a stage specific promoter that is activated in seed at the sensitive development stage of the seed, when the flag leaf sheath opens at the milky stage. It is at this stage in the development process that a seed is most susceptible to infection by *Fusarium* (Flannigan, 1987). For preventing formation of DHQR and providing a build-up of dihydroquercetin in transformed seeds, a preferred promoter is a testa-specific promoter, such as the leucocyanidin reductase gene promoter or a "DP3" gene promoter. The expression construct also utilizes additional regulatory DNA sequences *e.g.*, preferred codons, termination sequences, to promote efficient expression of gene encoding an inhibitory sequence such as an anti-sense DHQR gene.

The plants to be transformed are monocotyledonous plants, particularly the members of the taxonomic family known as the Gramineae. This family includes all members of the grass family of which the edible varieties are known as cereals. The cereals include a wide variety of species such as wheat (*Triticum sps.*), rice (*Oryza sps.*) barley (*Hordeum sps.*) oats, (*Avena sps.*) rye (*Secale sps.*), corn (*Zea sps.*) and millet (*Pennisettum sps.*). In the present invention, preferred family members are rice, corn, wheat and barley.

Various methods for direct or vectored transformation of plant cells, *e.g.*, plant protoplast cells, have been described, *e.g.*, in PCT application WO 95/14099 (incorporated herein by reference),

and are useful for obtaining a transgenic plant which produces seeds with resistance to a microbial pathogen, as described herein.

A promoter suitable for directing expression of a selectable marker used for plant transformation (*e.g.*, *npt*II) should operate effectively in plant hosts. One such promoter is the nos promoter from native Ti plasmids (Herrera-Estrella, *et al.*, 1983). Others include the 35S and 19S promoters of cauliflower mosaic virus (Odell, *et al.*, 1985), and the 2' promoter (Velten, *et al.*, 1984).

Introduction of the herein described cassette constructs may be carried out in any of the various ways known to those skilled in the art of plant molecular biology. See, for example, Wu and Grossman (1987), incorporated herein by reference. By transformation is meant alteration of the genotype of a host plant by the introduction of a nucleic acid sequence, and particularly a plant inhibitory sequence of the type described herein. The nucleic acid sequence need not necessarily originate from a different source, but it will, at some point, have been external to the cell into which it is to be introduced.

In one approach, the nucleic acid is mechanically transferred by microinjection directly into plant cells by use of micropipettes. Alternatively, the foreign nucleic acid may be transferred into the plant cell by using polyethylene glycol, which forms a precipitation complex with the genetic material that is taken up by the cell (Paszkowski, *et al.*, 1984).

Alternatively, the chimeric gene construct may be introduced into the plant cells by electroporation (Fromm, *et al.*, 1985). In this technique, plant protoplasts are electroporated in the presence of plasmids or nucleic acids containing the relevant genetic construct. Electrical impulses of high field strength reversibly permeabilize biomembranes, allowing the introduction of the plasmids. Electroporated plant protoplasts reform the cell wall, divide, and form a plant callus. Selection of the transformed plant cells with the transformed gene can be accomplished using phenotypic markers.

In a typical transformation methodology, the embryo and endosperm of mature seeds are removed to exposed scutulum tissue cells. The cells may be transformed by DNA bombardment or injection, or by vectored transformation, *e.g.*, by *Agrobacterium* infection after bombarding the scuteller cells with microparticles to make them susceptible to *Agrobacterium* infection (Bidney, *et al.* (1992).

A typical transformation protocol for rice generally follows the methods detailed generally in Sivamani, *et al.*, 1996; Zhang, *et al.*, 1996; and Li, *et al.*, 1993). Briefly, seeds are sterilized by standard methods, and callus induction from the seeds is carried out on MB media with 2,4D. During a first incubation period, callus tissue forms around the embryo of the seed. By the end of the incubation period, (*e.g.*, 14 days at 28°C) the calli are about 0.25 to 0.5 cm in diameter. Callus mass is then detached from the seed, and placed on fresh NB media, and incubated again for about 14 days

at 28°C. After the second incubation period, satellite calli develop around the original "mother" callus mass. These satellite calli are slightly smaller, more compact and defined than the original tissue, and are transferred to fresh media, while the "mother" calli are not transferred.

Calli to be bombarded are selected from 14 day old subcultures. The size, shape, color and density are all important in selecting calli for transformation. The calli typically should be between about 0.8 and 1.1 mm in diameter and should appear as spherical masses with a rough exterior.

Transformation is preferably by particle bombardment, as detailed in the references cited above. After the transformation steps, the cells are typically grown under conditions that permit expression of a selectable marker gene such as those described above. Alternatively, plasmid pEmuGN (Last, *et al.*, 1991), which directs high level expression of β-glucoronidase in monocots, can be used as an internal control to monitor the efficiency of gene transfer; substrate 5-bromo-4-chloro-3-indolyl is then used for histochemical staining of transformed tissue.

Preferably, the transformed cells are cultured under multiple rounds of selection to produce a uniform, stably transformed cell line.

Transformation of barley is preferably carried out as generally described in Jensen, *et al.*, 1996, and in Wan, *et al.*, 1994. Briefly, transformation plasmids on gold particles are delivered into immature embryos by particle bombardment. A preferred host for barley transformation is cv. Golden Promise. Reproducible transformation frequencies are typically obtained by growing the plants over a period of 60-80 days at temperature cycles of 18°C for 16h and 12°C for 8 h using high light intensities above about 120uE/m^2.

A preferred transformation protocol for barley is as follows. Immature embryos of about 1.5-2.5 mm in length are isolated from plants and grown under controlled conditions (day 0). The embryos are than bombarded with the target chimeric gene containing a selectable marker, *e.g.*, a herbicide resistance gene (day 1) and placed on selection media (day 2). During week 2, a first subcultivation is carried out and most immature embryos have exhibited growth. A second subcultivation is carried out at week 4, followed by a third subcultivation during week 6. During week 6, first callus is placed on shoot inducing medium. At about week 8, a fourth subcultivation is carried out and the first clones are placed on regeneration media. At week 10, a fifth subcultivation is carried out, and clones are placed on shoot inducing medium. By about week 12, a sixth subcultivation is conducted on shoot inducing and regeneration media; typically, the first green plantlets appear during this stage. At about week 14, a seventh subcultivation is carried out; this is followed during week 16 by an eighth subcultivation. During this period the first clones are then transferred to soil and allowed to grow for up until about month 7. DNA is then isolated from plant tissue and analyzed by PCR for the presence of the chimeric gene. By about month 8, the T_0 plants begin setting grains, and during month 9, the immature grains are harvested and the T1 embryos

germinated to establish the next generation. The T1 plants are grown to maturity, and the success rate of the transformation determined. Typical rates of transformation are about 1% transformants per isolated immature zygotic embryo.

Preferred methods of transforming wheat and sorghum are generally described in Nehra, *et al.*, 1994.

Another method of introducing the nucleic acid constructs of the invention into plant cells is to infect a plant cell, an explant, a meristem or a seed with Agrobacterium tumefaciens transformed with the segment. Under appropriate conditions known in the art, the transformed plant cells are grown to form shoots, roots, and develop further into plants. The nucleic acid segments can be introduced into appropriate plant cells, for example, by means of the Ti plasmid of Agrobacterium tumefaciens. The Ti plasmid is transmitted to plant cells upon infection by Agrobacterium tumefaciens, and is stably integrated into the plant genome and preferred methods for Agrobacterium-mediated transformation of corn and/or rice are generally disclosed in Horsch, *et al.* (1984) and Fraley, *et al.* (1983).

Ti plasmids contain two regions essential for the production of transformed cells. One of these, named transfer DNA (T DNA), induces tumor formation. The other, termed virulent region, is essential for the introduction of the T DNA into plants. The transfer DNA region, which transfers to the plant genome, can be increased in size by the insertion of the foreign nucleic acid sequence without its transferring ability being affected. By removing the tumor-causing genes so that they no longer interfere, the modified Ti plasmid can then be used as a vector for the transfer of the gene constructs of the invention into an appropriate plant cell, such being a "disabled Ti vector". All plant cells which can be transformed by Agrobacterium and whole plants regenerated from the transformed cells can also be transformed according to the invention so as to produce transformed whole plants which contain the transferred foreign nucleic acid sequence.

There are presently at least three different ways to transform plant cells with Agrobacterium: (i) co-cultivation of Agrobacterium with cultured isolated protoplasts, (ii) transformation of cells or tissues with Agrobacterium, or (iii) transformation of seeds, apices or meristems with Agrobacterium. The first method requires an established culture system that allows culturing protoplasts and plant regeneration from cultured protoplasts. The second method requires that the plant cells or tissues can be transformed by Agrobacterium, and that the transformed cells or tissues can be induced to regenerate into whole plants, while the third method requires micropropagation.

One preferred single strain Agrobacterium-based method of introducing the nucleic acid constructs of the invention into plant cells employs a super-binary vector system carrying two separate T-DNA segments (Komari, *et al.*, 1996), useful for the production of selectable marker-free transformants. Briefly, monocot cells are transformed with a super-binary vector carrying two

separate T-DNAs, where one T-DNA contains a selectable marker (*e.g.*, *nptII*, *hpt*) and the other contains a gene of interest, such as an inhibitory gene sequence as described herein. Initial drug-resistant transformants are analyzed to confirm integration and segregation of the T-DNAs (*e.g.*, by Southern hybridization or PCR). Progeny of the initial drug-resistant transformants, free from selection markers and carrying the non-selectable T-DNA carrying the gene of interest, are then identified; such progeny provide pathogen-resistant plants and seeds in accordance with the invention.

A. Growth of Transgenic Plants

For transgenics carrying a selectable marker gene, transgenic calli are cultured under conditions that favor selection of transformants in the presence of the substrate for the selectable marker, *e.g.*, phosphinotricin.

Plant regeneration from callus tissue is carried by standard methods, *e.g.*, as described in Evans, *et al.* (1983); and Vasil (1986). Representative growth conditions for transformed plants are described in Example 1.

Following introduction of the chimeric gene and growth of transformants, transformation of plant tissue can be confirmed by a variety of methods. Some exemplary methods for confirming transformation are described below.

a1. PCR ANALYSIS: When putative transformants are big enough, DNA is extracted from leaves and analyzed for the presence of the transgenes with appropriate primers (Jensen, *et al.*, 1996).

a2. SOUTHERN ANALYSIS: Transformation of each plant can be confirmed using Southern blot analysis of genomic DNA. Typically, total DNA is isolated from each transformant (*e.g.*, Schwarz-Sommer, *et al.*, 1984). The DNA is then digested with restriction enzyme, fractionated in 1% agarose gels and transferred to nylon filters (*e.g.*, HYBOND-N, Amersham) according to standard techniques. The blot is then probed, *e.g.*, with $_{32}$P-labelled DHQR cDNA as described.

b. NORTHERN ANALYSIS: RNA is isolated from immature kernels or testa layers as described in detail in Example 8, separated, *e.g.*, in a 1.2% agarose gel containing 2.2M formaldehyde, and blotted to a nylon filter, *e.g.*, Hybond-N, according to the supplier's protocol. Strand specific RNA probes are synthesized by phage T7 and T3 RNA polymerases from the *Ant18* cDNA clone and hybridized to

the RNA on the filter. This allows an estimation of the amount of endogenous sense and transgenic antisense mRNA for DHQR.

c. HPLC EXTRACTS: Extracts from immature kernels of transgenic plants are analyzed by High Performance Liquid Chromatography, as described in Example 5, in order to detect the presence and/or absence of particular flavonoids such as dihydroquercetin and catechin. The presence of dihydroquercetin and absence of catechin are indications that formation of DHQR is blocked in transgenic plants produced as described above.

d. DIHYDROFLAVONOL REDUCTASE (DFR) ASSAY: Enzyme extracts from immature kernels of transformed plants can also be assayed for production of dihydroquercetin reductase (Kristiansen, 1986). The absence of the enzyme or its reduced presence indicates that expression of the *Ant*18 gene is inhibited in transformed plants.

B. Monitoring for *Fusarium* Growth

Plants transformed by the methods described herein exhibit extreme resistance to infection by *Fusarium*. Infection of such transformed plants or seeds by *Fusarium* can be monitored as follows.

In the early development stage, plots in the field or greenhouse (*e.g.*, barley, wheat, corn, rye, millet, corn, etc.) can be effectively inoculated by spraying with a suspension of the sickle shaped, hyaline, multiseptate macroconidia generated by *Fusarium* cultures on potato dextrose agar (Haikara, 1983). Plant genotypes can then be screened for susceptibility to *Fusarium* with this method.

One of the most specific and sensitive tests for the presence of *Fusarium* is the analysis of extracts from single grains or larger samples with antibodies produced against extracts of mycelium in an enzyme biotin streptavidin linked immunosorbent assay (EBStrALISA). These antibodies recognize *Fusarium*, and also antigens of *Helminthosporium*, *Alternaria* and some strains of *Aspergillus* (Vaag, 1991). The cross reactions can, however, be suppressed by preincubation of the antibodies in the coat layer with antigens of culture substrates of the two fungi. Using this technique, the development of the *Fusarium* fungus in maturing grain and in malting grains during steeping and germination can be monitored with great precision (Vaag, 1991).

To determine the extent of infection of seed transformants blocked in DHQR production versus wild-type grain, the following experiment is carried out.

Barley seeds (both transformed and wild type) are soaked in water for 3 days at 25°C. Germinated seeds are planted in plastic boxes (1/10 of nursery box) containing 400 ml of *Fusarium*-contaminated soil. The seeds are then covered with 100 ml pasteurized soil and maintained in a moisture chamber (27°C, 100% relative humidity) for 3 days. Boxes are then moved to the greenhouse where they are typically subirrigated daily. Disease control evaluations as described above are made 7-10 days after seeding by counting healthy versus infected seedlings.

The transformation methods, chimeric genes, vectors, transgenic plants, cells and seeds described herein are useful for providing a method for essentially preventing growth of *Fusarium* in such transformants. In the method of the present invention, resistance to *Fusarium* is imparted by utilizing techniques (*e.g.*, inhibitory gene sequences such as point mutants and antisense constructs; overexpression of an upstream enzyme resulting in increased production of dihydroquercetin, or co-suppression) which block the formation of a particular metabolic enzyme, DHQR, to provide an *in-vivo* accumulation of the potent antimycotic compound, dihydroquercetin, within transformed seeds.

V. Mold-Resistant Seeds and Seed Products; Heterologous Protein Production in Seeds

As described above, the present invention is directed to a method for producing transformed seeds and seed products which are essentially free of contaminating pathogens. By seed product is meant the seed grain itself or parts thereof, as well as any seed extract, flour, malt or malt product, or foreign protein produced by the seed, which, as a result of the technology described herein, is essentially free of potentially harmful microbial contaminants.

Monocot seeds are susceptible to infection by molds, including pathogenic fungal infection. Many molds and fungi produce mycotoxins, which when ingested can cause toxic or carcinogenic symptoms in humans and animals. (Butler, 1974; Smith and Hacking, 1983). Mycototoxognic fungi have been isolated from many foods which are subject to mold spoilage. The main genera of fungi associated with mycotoxin production include species of *Aspergillus, Penicillium, Fusarium, Clavicps, Alternaria, Pithomyces, Stachybotrys and Phoma*. (Wainwright, 1992.)

Mycotoxins are not produced in the absence of mould contamination, although a given toxin may persist in a food or foodstuff in the absence of the fungus. A single species of toxin may also produce more than one toxin, while an individual mycotoxin may be produced by a number of different fungi.

Cereals are highly susceptible to fungi infection and resulting mycotoxin contamination, both when growing in the field and when stored and processed. These infections and resulting contamination can be a problem in processing of cereal grain seeds. (See, Flannigan, 1996)

So for example, during the malting stage in particular, dormant fungi and other microorganisms are activated. Microorganisms that have been associated with monocots during or

after the malting stage include *Aureobacterium, Cladosporium, Mucor, Penicillium, Fusarium, Aureobasidiumpullulans, Trichosporon, Alt. alternata, Rhizopus, Candida, Cryptococcus, Richia, Rhodotorula, Pseudomonas*, and *Bacillus* (Flannigan, 1996).

Thus, as can be seen, the present invention provides a means for minimizing, and essentially eliminating the problem of contaminating microorganisms in seeds and seed products.

Thus, it follows that another important aspect of the invention is directed to a method for producing recombinant heterologous (foreign) proteins in seeds from a transformed monocot plant, where the recombinant proteins are substantially free of contaminating mycotoxins.

To address this problem, a method is provided for producing recombinant proteins in seeds of a transformed monocot plant, where fungal infection is inhibited in seeds producing the protein. This aspect of the invention provides a method for producing recombinant proteins that are essentially free of contaminating mycotoxins, thereby reducing the number of post-protein production purification steps required to obtain a purified protein product.

In practicing the method, fungal infection is inhibited by genetically altering a monocot plant to achieve elevated seed levels of a secondary metabolite effective to inhibit fungal infection of seeds from the plant. The plant may be genetically altered by mutating a gene encoding an enzyme that converts the secondary metabolite to another product that is less toxic to the fungus, to produce a mutant incapable of producing the enzyme in seeds of the plant. Examples of mutant genes include barley *ant*18 mutants, and A1 gene mutants for the A1 corn and rice genes. Alternatively, the plant may be genetically altered by transforming the monocot plant with a chimeric gene having (i) a transcriptional regulatory region induced during seed development, and (ii) an inhibitory gene sequence operably linked to the transcriptional regulatory region for induction of an inhibitory transcript RNA capable of inhibiting expression of a gene encoding an enzyme that normally converts the intermediate secondary metabolite to another metabolite that is less toxic to the fungus (*e.g.*, a DHQR gene encoding dihydroquercetin reductase).

The method further includes stably transforming the plant with a chimeric gene having (i) a transcriptional regulatory region inducible during seed germination, (ii) a first DNA sequence encoding the heterologous protein, and (iii) a second DNA sequence encoding a signal polypeptide. The second DNA sequence is operably linked to the transcriptional regulatory region and the first DNA sequence, while the signal polypeptide is in translation-frame with the protein and is effective to facilitate secretion of the protein across the aleurone or scutellar epithelium layer of seeds from the altered, transformed plant into the endosperm.

Seeds are then obtained from the altered, transformed plant. The seeds are then malted under conditions favoring heterologous protein production in the seeds, and the heterologous protein is

isolated from the seeds. The protein is substantially free of mycotoxins, or has a substantially reduced burden of mycotoxins by virtue of the fungal infection inhibition.

Further details regarding this aspect of the invention will now be described, and are illustrated in Example 9 for production of α-antitrypsin in malted barley seed from transformed *ant*18 barley plants.

A. Transformation With a Chimeric Gene Encoding an Exemplary Heterologous Protein

1. Transformed plant cells. Plant cells or tissues derived from monocot plants are transformed with expression vectors as described above containing a chimeric gene coding for a heterologous protein, *e.g.*, a therapeutic protein such as α-antitrypsin (ATT), erythropoietin (EPO), tissue plasminogen activator (t-PA), urokinase and prourokinase, growth hormones, cytokines, factor VIII, epoetin-α, granulocyte colony stimulating factor, and vaccines, using a variety of standard techniques as described above (*e.g.*, electroporation, protoplast fusion or microparticle bombardment).

The expression construct includes a transcription regulatory region (promoter) whose transcription is preferably upregulated by the presence or absence of a small molecule, such as the reduction or depletion of sugar, *e.g.*, sucrose, in culture medium, or in plant tissues, *e.g.*, germinating seeds. For this aspect of the invention, particle bombardment is a preferred transformation procedure.

The construct also includes a gene encoding a heterologous protein, *e.g.*, a therapeutic or non-therapeutic protein as above, in a form suitable for secretion from plant cells. The gene encoding the recombinant protein is placed under the control of a metabolically regulated promoter, such as described above. Metabolically regulated promoters are those in which mRNA synthesis or transcription, is repressed or upregulated by a small metabolite or hormone molecule, such as the rice RAmy3D and RAmy3E promoters, which are upregulated by sugar-depletion in cell culture. For protein production in germinating seeds from regenerated transgenic plants, a preferred promoter is the rice RAmy 1A promoter or the barley HV18 promoter, both of which are up-regulated by gibberellic acid during seed germination (see Example 9).

The expression construct also utilizes additional regulatory DNA sequences *e.g.*, preferred codons, termination sequences, to promote efficient translation of the gene encoding the protein, as will be described.

B. Production of Recombinant Proteins

This section briefly describes methods for producing recombinant proteins in plant (*e.g.*, seed) cells transformed in accordance with this aspect of the invention.

1. Foreign protein production in germinating seeds. In a preferred method for producing the heterologous protein, monocot cells transformed as above are used to regenerate plants, seeds from the plants are harvested and then germinated, and the recombinantly-produced protein is isolated from the germinated seeds which are substantially free from fungal infection.

2. Seed Germination Conditions: Malting. The transgenic seeds obtained from the regenerated plants are harvested, and prepared for germination by an initial steeping step, in which the seeds are immersed in or sprayed with water to increase the moisture content of the seed to between 35-45%. This initiates germination. Steeping typically takes place in a steep tank which is typically fitted with a conical end to allow the seed to flow freely out. Optionally, compressed air is added to oxygenate the steeping process. The temperature is controlled at approximately 22°C, depending on the seed.

After steeping, the seeds are transferred to a germination compartment which contains air saturated with water and is under controlled temperature and air flows. The typical temperatures are between 12-25°C and germination is permitted to continue for about 3 to 7 days.

Where the protein coding gene is operably linked to a inducible promoter requiring a metabolite such as sugar or plant hormone, *e.g.*, 2.5 to 100 μM gibberellic acid, this metabolite is added, removed or depleted from the steeping water medium and/or is added to the water saturated air used during germination. The seed absorbs the aqueous medium and begins to germinate, thereby expressing the heterologous protein (*e.g.*, ATT). The medium may then be withdrawn and the malting begun, by maintaining the seeds in a moist temperature controlled aerated environment. In this way, the seeds may begin growth prior to expression, so that the expressed product is less likely to be partially degraded or denatured during the process.

More specifically, the temperature during the imbibition or steeping phase will be maintained in the range of about 15-25°C, while the temperature during the germination will usually be about 20°C. The time for the imbibition will usually be from about 2 to 4 days, while the germination time will usually be an additional 2 to 10 days, more usually 3 to 7 days. Usually, the time for the malting does not exceed about ten days. The period for the malting can be reduced by using plant hormones during the imbibition, particularly gibberellic acid.

To achieve maximum production of mycotoxin-free recombinant protein from malting, the malting procedure may be modified to accommodate de-hulled and de-embryonated seeds. In considering plants transformed with a chimeric gene under the control of either a RAmy3D or RAmy1A promoter, in the absence of sugars from the endosperm, there is expected to be a 5 to 10 fold increase in RAmy3D promoter activity and thus expression of heterologous protein.

Alternatively, when embryoless-half seeds are incubated in 10mM $CaCl_2$ and 5 μM gibberellic acid, there is a 50 fold increase in RAmy1A promoter activity.

C. Isolation of Recombinant Protein

After optimum germination and expression of the protein gene have been achieved, the seeds are mashed (for example, by gentle grinding) to disrupt tissues and remove the hulls from the seeds. The seed mash is suspended in a protein extraction buffer which typically contains protease inhibitors, reducing agents and a buffering agent (such as, "TRIS" or sodium or potassium phosphate), and has a preferred pH of between 5-6.

The mash is agitated or stirred to ensure that all secreted protein is freed from tissues and cells. Large particulate matter, such as hull, plant tissues, and other debris are then removed by filtration or centrifugation. The supernatant is collected and chilled to reduce proteolysis of the recombinantly produced protein.

The supernatant is subjected to various purification schemes used in the wet-milling industry (*e.g.*, hydrocloning and ion exclusion chromatography) to remove unwanted proteins and to concentrate the product. Alternatively, ammonium sulfate precipitation can also be used to concentrate certain proteins such as AAT.

Affinity- and ion-exchange chromatography can also be used to purify the mycotoxin-free recombinant protein away from other proteins in the supernatant.

Alternatively, after transportation of the transgenic seeds to a malting facility (malthouse), the seeds are dehulled and de-embryonated (*i.e.*, mechanical separation of the embryos and endosperm portions of the seed). The embryos and endosperms are separately soaked (steeped) in water for 48 hours. The seeds are treated as described above and the separated embryos are treated as follows. Expression driven by an inducible promoter such as the RAmy3D promoters is induced in the absence of sugar and/or by the addition of chemicals, such as a plant hormone, *e.g.*, absissic acid.

After optimum germination and expression of the protein gene have been achieved, the embryo and endosperm portions are mixed and then mashed (*i.e.*, gentle grinding) to disrupt seed tissues. The mash is then treated as above for purification of the protein which is substantially free of mycotoxin contaminants.

Materials and Methods

A. General Methods

Biological reagents were typically obtained from commercial vendors, unless otherwise indicated.

Standard molecular biology techniques were employed as described in Ausubel, *et al.*, 1992; Sambrook, *et al.*, 1989; Maniatis, *et al.*, 1982.

B. Plant Material

The barley (*Hordeum vulgare, L.*) cultivars and corresponding isolated proanthocyanidin-free mutants (*ant*): Grit, *ant*22-1508, *ant*25-264, *ant*26-485, *ant*27-148, Gula, *ant*18-159, *ant*-161, *ant*18-162, *ant*18-164; Alf, *ant*19-109, were prepared as described in Jende-Strid, 1995.

Barley plants were grown in a greenhouse at 15°C with a 16 h light and 8 h dark period. Caryopses at various times after anthesis were analyzed.

The legumes *Lotus uliginosus* and *Lotus japonicus* were grown under the same conditions as the barley plants. *Robinia pseudacacia* leaves and *Ribes sanguineum* flowers were collected from the garden at the Carlsberg Research Centre from April to June 1995. The leaf and flower tissue was immediately frozen in liquid nitrogen.

C. Crude Plant Extracts

Testa/pericarp layers (0.5 g) were isolated 17-20 DAA (days after anthesis) from barley grains as described above. One ml of water was added, and the tissue was homogenized using a polytrone. The sample volume was reduced to 100 µl *in vacuo* to minimize dilution.

R. sanguineum flowers, *R. pseudacacia*, *L. japonicus* and *L. uliginosus* leaves (0.5 g) were frozen in liquid nitrogen and homogenized with a polytrone.

D. DFR Assay

Enzyme extracts from 8-13 day old developing barley grains (wild type and mutants) were prepared and an assay for DFR was performed using (+)-[^{14}C]-DHQ as substrate for NADPH-dependent conversion to (+)-2,3 *trans*-3,4-*cis*-leucocyanidin as described in Kristiansen (1986).

E. Flavonoid Standards

Tannic acid, dihydroquercetin (DHQ), (Sigma Chemical Co., St. Louis, MO), catechin, epicatechin, dihydromyricetin, cyanidin chloride (Apin chemicals), procyanidin B3 and C2 isolated from wild type barley (Carlsberg Research Laboratory, Denmark), and sainfoin leaf polymer (Agriculture Canada) were used as flavonoid standards.

The following examples illustrate, but in no way are intended to limit the present invention.

Examples

Example 1

SODIUM AZIDE INDUCED TREATMENT OF BARLEY GRAINS

Barley mutants were prepared by sodium azide-induced mutagenesis as described in Olsen, *et al.*, 1993). A summary of 29 known *ant* gene loci in barley is provided in Jende-Strid, the contents of which is herein incorporated by reference (1995).

Preparation and analysis of representative *ant*18 mutants, *ant*18-159, *ant*-161, *ant*18-162, *ant*18-164 barley mutants, is described below.

A. Mutagenesis of Barley Grains

Barley (*Hordeum vulgare L.*) cv. Gula grains were presoaked for 15 h in distilled water at 5°C and then treated in an oxygenated solution of 1 mM NaN_3 at pH 3.0 for 2 h. The grains were rinsed and planted in the field. The M_2 progenies were planted the following year and the homozygous mutants *ant*18-159, *ant*-161, *ant*18-162, *ant*18-164, were selected.

B. Plant Growth Conditions

Grains were planted in potting compost and grown in a growth chamber under controlled conditions for 6 d at 22°C in continuous light followed by 9 days of incubation with a 16 h photoperiod at 15°C and 8 h in the dark at 10°C.

C. Cloning of Genomic DNA

Barley genomic DNA was isolated from 0.1 g of wild type or mutant leaf tissue according to standard techniques (Edwards, *et al.*, 1991) and dissolved in 400 microliters of water. An aliquot of 70 microliters was digested with 200 units of *Hind*III for 15 minutes before desalting and gel chromatography using Croma Spin-1000 columns (Clontech, Palo Alto, CA). Twenty microliters of purified DNA was used as a template for PCR (Mullis, 1987; Mullis, *et al.*, 1987). By using phosphorylated oligonucleotide primers (30-mers) identical to the *ant*18 gene sequence (SEQ ID NO:1; Fig. 10, Kristiansen and Rohde, 1991), four partially overlapping fragments spanning 3176 bp of the chromosome segment were amplified from each mutant and wild type gene. The products contained the following endpoints: -1283 to -606 (fragment 1), -723 to 145 (fragment 2), -243 to 919 (fragment 3) and 853 to 1893 (fragment 4).

Amplification reactions were carried out for 25 cycles using denaturation at 94°C for 1 min, annealing at 60°C for 2 minutes, and primer extension at 72°C for 2 minutes before separation of the amplified products by agarose gel electrophoresis. DNA fragments of interest were purified from the gel by using Prep-A-Gene matrix (Bio-Rad, Richmond, CA), ligated into pUC18 linearized with *Sma*I

(Pharmacia, Piscataway, NJ), and cloned in *E. coli* DH5α cells (GIBCO/BRL, Gaithersburg, MD). Plasmid DNA for sequencing was purified with Qiagen columns.

D. Nucleotide Sequence Determination

Double stranded plasmids containing cloned gene fragments were sequenced with gene-specific primers using a model 373A DNA sequencer (Applied Biosystems, Inc., Foster City, CA) according to the manufacturer's instructions.

1. *ant*18-159 mutant allele: DNA sequence analysis revealed five base substitutions in the *ant*18-159 gene. Three substitutions, A-203 to G, A-596 to G, and T-931 to C, were identified in the 5' end of the promoter sequence of *ant*18-159, and two transitions were identified in the protein coding region: G-2163 to A, which introduces a basic Lys residue for an acidic Glu at position 176; and A-2292 to G, which substitutes Ala for the conserved Thr-214.

2. *ant*18-162 mutant allele: Mutations T-234 to A and T-459 to C were identified in the promoter region of this gene, and G-2947 to A was identified in the 3' non-coding region. Two mutations were identified in the DFR coding region, T-1434 to C and A-1849 to T. These mutations alter conserved residues, Val-32 to Ala and Lys-101 to Met, respectively.

3. *ant*18-164 mutant allele: Two silent transitions were identified in the protein coding region, C-1696 to T, and T-1940 to C. Additional identified mutations included two base substitutions, G-1374 to A and T-1386 to A, which change Gly-12 to Glu and Phe-16 to Tyr in the translated product, respectively. Two additional mutations, A-69 to B and A-180 to G at the 5' end of the promoter, and A-2080 to G in the center of intron 3, were also identified.

4. *ant*18-161 mutant allele: Four base substitutions were identified for the *ant*18-161 mutant: T-70 to C, A-1406 to G, G-2031 to A and T-2032 to C. The last two transition mutations eliminate the obligatory GT donor splice site for excision of the third intron in the primary transcript of the *Ant* 18 gene and lead to the elimination of mature mRNA.

Example 2

IN VITRO INFECTION OF BARLEY GRAINS WITH *FUSARIUM*

A. *Fusarium* Strains

The following *Fusarium* strains were used: *F. poae*, *F. culmorum* and *F. graminearum* (Carlsberg Research Laboratory, Denmark).

The strains were grown at room temperature for 7-10 days on CZID agar plates. One liter medium contained 35 g Czapek-Dox Broth (Difco Laboratories, Detroit, MI), 50 mg chloramphenicol (Sigma Chemical Co., St. Louis, MO), 1 ml trace metal solution (10 mg $ZnSO_4 \cdot 7H_2O$ and 5 mg $CuSO_4 \cdot 5H_2O$ in H_2O), 1 ml Dichloran solution (0.2 mg 2,6-dichloro-4-nitroalanine (Fluka Chemie, Buchs, Switzerland) in 1 ml ethanol, 15 g agar (Difco) and deionized H_2O to 1 l. The autoclaved medium was cooled to 50°C, whereafter 10 ml tetracycline and 1 ml Iprodion solution were added. Five mg oxytetracycline (Sigma Chemical Co., St. Louis, MO) were dissolved in 10 ml H_2O and passed through a sterile 0.2 μm mesh filter and 1 ml Iprodion solution (6 mg Iprodion (Rhône-Poulenc Agro-Chemie)) were dissolved in 1 ml H_2O and passed through a 0.2 μm mesh sterile filter for sterilization (Abildgren, *et al.*, 1987). Spores were harvested by carefully washing them off from surface of the plate using 10 ml of sterile water and the titer was determined using a counting chamber.

B. *In vitro* Infection of Barley Grains

1. Infection by *F. poae*, *F. culmorum* and *F. graminearum*: Ten grams of barley grains from wild type (Grit, Triumph and Alf) cultivars and proanthocyanidin-free barley mutants, *ant*22-1508, *ant*25-264, *ant*26-485, *ant*27-489, *ant*28-484, *ant*29-2110, *ant*13-152, *ant*17-148, *ant*18-159, and *ant*19-109, were collected at 20 days after anthesis. Seeds were surface sterilized by washing in 1% (v/v) hypochlorite solution for 20 min and then rinsed 6 times in 100 ml of sterile water for 5 minutes. 1 ml spore-suspensions with 2×10^3, 4×10^5 or 2×10^7 spores/ml of *F. poae*, *F. culmorum* or *F. graminearum* were added per sample and carefully mixed by inverting the tube several times. Grains were distributed on CZID plates (⌀ 9 cm) and incubated for one week.

Figs. 3A-3D illustrates the different degrees of pathogenicity of *F. culmorum* on both mutant and wild type genotypes (Fig. 3A = Triumph, Fig. 3B = *ant*13-152, Fig. 3C = *ant*17-148, and Fig. 3D *ant*18-159).

2. Infection by *F. graminearum*. In a separate experiment, 20 barley grains from each wild type and proanthocyanidin-free mutant (25 DAA) were infected as described above with *F. graminearum* (2×10^7 spores/plate with 16 kernels) and incubated for one week.

C. Histological Investigations of *Fusarium* Infected Barley Grains

1. Embedding Kernels for Sectioning. Kernels were fixed with Jung freeze glue (Leica Instruments), 5-10 μm thick sections prepared on a freeze cryotome and fixed onto slides using Eukitt.

Grains were fixed for 6 h in 2% (v/v) formaldehyde and 2.5% (v/v) glutaraldehyde in 60 mM potassium buffer at pH 7.0 for 4 h. Each kernel was divided into four pieces and fixed for additional 24 h. Tissues was washed three times 30 min in 60 mM potassium phosphate buffer (pH 7), dehydrated in a graded ethanol series (30, 50, 77, 90, 96 and 100% ethanol), infiltrated and embedded in Spurr's resin (Sigma Chemical Co., St. Louis, MO) with subsequent polymerization at 70°C overnight. Cross sections, 3-5 μm thick, were cut with glass knives on a Reichert OM U3 microtome, baked onto an object glass at 90°C, stained with fuchsin and toluidine blue (Aslop, 1974) and mounted in Eukitt.

2. Microscopy. Sections were analyzed in a Zeiss Axioplan NMC100 Universal Microscope fitted with a camera and photographed, using either polarized light, phase-, interference- or fluorescence-contrast.

Histological analyses of *F. colmorum in vitro*-were carried out on Triumph, *ant*13-152, *ant*17-148 and *ant*18-159 infected barley grains one week following inoculation.

Fluorescence microscopy (blue excitation filter) was used to examine *ant*13-152 cross-sections embedded in Spurr's resin and stained with toluidine-blue (mag. 550 × and 6200 ×) 1 week following inoculation. Figs. 4C-4D show cryo-sections of infected Triumph kernels, Fig. 4C, after 1 week infection (mag. 1300 ×); Fig. 4D, after 2 weeks (mag. 900 ×); Interference-contrast microscopy was employed to examine cryo-sections of *ant*17-148 kernels 1 week post infection (mag. 1050 ×) and cryo-sections of *ant*18-159 kernels 2 weeks post-infection (mag. 2100 ×).

The results of this analysis indicated that in contrast to all other genotypes, hyphae did not penetrate through the seed coat (testa layer) of *ant*18-159, even after 2 weeks of incubation with the highest spore concentration tested.

3. Results: Resistance to Infection by *Fusarium culmorum*: Triumph (Fig. 3A) was less infected than *ant*13-152 (Fig. 3B) and *ant*17-148 (Fig. 3C), but sustained more attack than *ant*18-159 (Fig. 3D), which revealed outstanding resistance. Kernels of *ant*13-152 were quite infected already after 4 days incubation and completely overgrown after one week. Mutant *ant*17-148 was slightly less infected than *ant*13-152, whereas only little growth of mycelium could be seen on the ends of *ant*18-159 kernels under the microscope. After 5-6 days of incubation, the CZID agar on which *ant*13-152 and *ant*17-148 grains were positioned, turned dark red due to the aurofusarin pigment

secreted by the fungus. This color formation was observable only after 5-6 weeks in the agar under the Triumph and *ant*18-159 grains.

4. Resistance to *F. graminearum*: A comparison of all proanthocyanidin-free mutants and wild types investigated after one week of incubation with *F. graminearum* revealed *ant*13-152, *ant*17-148, *ant*22-1508 and *ant*25-264 to be especially prone to strong infection. Mutants *ant*26-485 and *ant*27-489, *ant*28-484 and *ant*29-2110 were attacked less, but significantly more than the corresponding wild types. Kernels of *ant*18-159 showed consistently more resistance to *Fusarium* attack than any of the wild types tested.

The length of time it took for *ant*18-159 kernels to become overgrown with *F. culmorum* mycelium in comparison with *ant*13-152 kernels was also investigated. While *ant*13-152 was strongly infected and overgrown with mycelia after one week incubation, 6 weeks were necessary for *ant*18-159 to be overgrown to the same extent.

Repetitions of infection experiments with *F. poae*, *F. graminearum* or *F. culmorum* at different spore-concentration, supported these findings.

The sensitivity of both mutant and wild-type barley towards *Fusarium* infection is summarized in Table 2.

Table 2

Sensitivity of Proanthocyanidin (PA)-Free Barley Mutants and Wild Types Towards *Fusarium* Infection in Relation to the Presence of Anthocyanins, Proanthocyanidins (PAs) and Flavonoids Accumulated in the Testa Layer

Genotype	Anthocyanin	PA	Flavonoids Occurring in the Grain	Inhibition
Triumph	+	+	anthocyanins, monomeric, dimeric and trimeric PAs	++
*ant*13-152	-	-		-
*ant*17-148	-	-	flavanone-homoeriodictyol, flavon-chrysoeriol	-
*ant*18-159	-	-	dihydroquercetin	+++
Alf	+	+	anthocyanins, monomeric, dimeric and trimeric PAs	++
*ant*19-109	+	-	anthocyanins, small amounts of catechin and Procyanidin B3	+
Grit	+	+	anthocyanins, monomeric, dimeric and trimeric PAs	++
*ant*22-1508	-	-	flavanone-homoeriodictyol, flavon-chrysoeriol	-
*ant*25-264	(+)	-	anthocyanin	-
*ant*26-485	+	-	anthocyanin trace amounts of catechin	+
*ant*27-489	+	-	anthocyanin trace amounts of catechin	+
*ant*28-484	+	-	anthocyanin	+
*ant*29-2110	+	-	anthocyanin trace amounts of PA	+

Example 3

PROANTHOCYANIDIN ACCUMULATION IN DEVELOPING BARLEY TESTA LAYERS

Testa layers were isolated from wild type barley (Triumph, Grit, Alf) and proanthocyanidin-free mutants, to study the accumulation of proanthocyanidins during grain development.

A. Isolation of Testa Layers

Testa layers were isolated from barley caryopses between 10 and 35 days after anthesis (DAA). Awns and glumes were peeled off, whereafter the translucent epidermis layer, and the green pericarp including the adhering chlorophyll/anthocyanin containing crosscell-layers were removed. The thin testa layer was then separated from the aleurone layer.

B. Vanillin/HCl Staining

Seeds or testa layers were placed in a 1% (w/v) vanillin-6 M HCl solution for 5-10 min, which stains proanthocyanidins and catechins bright red (Sakar and Howarth, 1976).

C. Wild-Type Barley

At 10-15 DAA, the cytosol contained numerous intensely red stained globules (probably consisting of proanthocyanidin filled vacuoles). The caryopsis had differentiated the pericarp into its well defined layers of cross and tube-cells, the starch contained mesocarp and the heavily cutinized epidermal cells. At 18-20 DAA, the testa-cells were filled with the proanthocyanidin containing vesicles and at 25 DAA these had fused to larger compartments with a crystalline structure. In the period of 35-40 DAA cracks appeared in the proanthocyanidin deposits.

D. Proanthocyanidin-free Mutants

Testa layers isolated from proanthocyanidin-free barley mutants developed the granular structure and vacuoles, but lacked the intense red color after vanillin-HCl staining. The testa layers (20 DAA) isolated from the mutants *ant*13-152, *ant*17-148, *ant*18-159, *ant*22-1508, *ant*25-264 and *ant*28-484 did not stain red with vanillin-HCl, confirming the complete absence of proanthocyanidins.

A pale reddish color of the granules was observed in *ant*19-109, *ant*26-485, *ant*27-489 and *ant*29-2110. These mutants synthesize normal levels of anthocyanin and small amounts of catechin. The pale reddish color was attributed to catechin. Mutant *ant*19-109 formed extensive yellow-brown deposits in cells scattered among the pale red testa-cells.

Example 4

EFFECT OF EXTRACTS OF BARLEY TESTA LAYERS ON GROWTH OF *FUSARIUM* INHIBITION ASSAYS

A. Inhibition by Flavonoid Standards

F. culmorum spores 6×10^5/ml were spread onto a CZID plate and the excess liquid evaporated. Then 1 mg of each flavonoid standard dissolved in 2 μl methanol and 8 μl water was applied in wells on the surface of CZID plates and these were incubated for one week at room temperature under UV light.

B. Inhibition by Dihydroquercetin

DHQ (10, 25, 50, 100, 200 or 500 mg/l) was mixed with CZID medium before solidification (55°C) and poured into petri dishes. The solidified plates were seeded with 5×10^7 spores of *F. culmorum* and incubated as described above.

Representative results are as follows. After one week, *Fusarium* started to grow on plates containing 10, 25 and 50 mg/l DHQ, while very little growth occurred on plates with higher concentrations. After two weeks extensive growth had occurred on media with 10, 25, 50 mg/l DHQ and white mycelium was observed on plates containing 100, 200, 500 mg/l of dihydroquercetin. Inoculated plates with ≤ 50 mg/l of DHQ turned increasingly reddish after 3 weeks incubation. Even after six weeks, only white mycelium was observed on plates with DHQ concentrations higher than 100 mg/l, and no spores developed.

C. Inhibition by Testa Pericarp Extracts

Testa/pericarp extracts from wild type barley grains (Triumph, Grit, Alf) and barley mutants (*ant*13-152, *ant*17-148, *ant*18-161, *ant*19-109, *ant*26-485) were applied onto CZID plates containing either 3×10^3, 1×10^5 or 1×10^7 *F. culmorum* spores/plate and incubated for 10 days.

After 5 days *Fusarium* growth became visible, especially on plates with high spore concentrations. Mutant *ant*18-159 extracts exhibited a more pronounced inhibition zone than those of the three wild types. This difference was enhanced at lower spore concentrations.

D. Inhibition by Other Proanthocyanidin-Rich Tissue

Homogenized tissue extracts (0.5 g) from flowers of *R. sanguineum* and from leaves of *R. pseudacacia*, *L. japonicus* and *L. uliginosus* were mixed with 15 ml CZID agar (55°C) and plates were poured. After solidification, plates were seeded with 5×10^7 spores of *F. culmorum* and incubated for one week.

No growth of *Fusarium* was observed on CZID plates containing *R. sanguineum* flower extract, except for small colonies of white mycelium. Also *R. pseudacacia* and *L. uliginosus* leaf extract limited fungal growth. *L. japonicus* leaf extract (proanthocyanidin-free extract; control) did not inhibit fungal growth; after 5 days of incubation the plate was completely overgrown with *Fusarium*.

The inhibitory effect of flavonoids and related compounds, flavonoid standards, and monomeric and polymeric compounds of several plants against *Fusarium* is provided in Table 3.

Table 3

Inhibition of *F. culmorum* Growth by Flavonoid Standards, Monomers and Polymers from Plant Extracts

Flavonoid/Compound	Inhibition
Tannic Acid	++
Catechin	++
Epicatechin	++
Dihydromyricetin	+
Dihydroquercetin	+++
Cyanidin Chloride	-
Procyanidin-B3	+
Procyanidin-C2	+
Onobrychis viciifolia (leaves)	
Polymer	+
Robinia pseudacacia (leaves)	
Monomers	+++
Polymers	++
Ribes sanguineium (flowers)	
Monomers	+++
Polymers	++
Lotus uliginosus (leaves)	
Monomers	++
Polymers	+
Lotus japonicus (leaves)	
Monomers	-

- no inhibition, + little inhibition, ++ inhibition, +++ strong inhibition

Example 5

PURIFICATION OF FLAVONOID MONOMERS AND POLYMERS

Flavonoid monomers and polymers were purified according to Koupai-Abyazani, *et al.* (1992). Finely ground testa/pericarp tissue (5 g) from Triumph, *ant*13-152, *ant17-148, ant*18-159,

flowers from *R. sanguineum*, leaves from *R. pseudacacia*, *L. japonicus* or *L. uliginosus* were extracted with 4 × 10 ml 75% v/v aqueous acetone containing 0.1% (w/v) ascorbic acid. The acetone was removed under reduced pressure at temperatures < 30°C. The aqueous solution was filtered through a plug of gauze, and the filtrate was extracted with chloroform (3 × 15 ml).

After phase separation, the aqueous phase was extracted with ethyl acetate (4 × 25 ml), and the ethyl acetate soluble fractions, containing the monomeric compounds, were pooled. The resulting aqueous phase containing proanthocyanidins was mixed with the same volume of methanol and applied on a Sephadex LH-20 column (15 cm × 4 cm; Pharmacia, Piscataway, NJ). After binding of the proanthocyanidins, the column was washed with 600 ml of 50% (v/v) aqueous methanol and eluted with 75% (v/v) aqueous acetone (500 ml). Acetone was evaporated under vacuum and the solutions lyophilized.

Monomeric flavonoids from Triumph, *ant*13-152, *ant*17-148, *ant*18-159 were separated by preparative HPLC on a (Bondapack phenyl column, (30 cm × 3.9 mm; Waters, Milford, MA) using a linear gradient from 2 to 10% v/v acetic acid in 60 min at a flow rate of 2 ml/min. The elution of the monomers was monitored spectrophotometrically at 280 nm. Between each run, 1 h elution with 10% v/v acetic acid was necessary to elute all flavonoids. Compounds were identified based on their retention times (RT) compared with known standards. Pooled barley flavonoid samples from different HPLC runs were dried and dissolved in 50 μl of 10% v/v methanol.

Figs. 1A-1F are HPLC profiles of monomeric flavonoids extracted from (A) Triumph, (B) *ant*18-159 transformed barley, (C) *ant*13-152 transformed barley, (D) *ant*17-148 transformed barley; (E) DHQ standard; and (F) catechin standard.

Example 6

Identification of Flavonoid Monomers from *Ribes Sanguineum* Flowers

HPLC was carried out on a Waters (Milford, MA) M600E system equipped with a multisolvent delivery system, an M700 autosampler and a programmable 991 photodiode array detector (PDA), using the PDA software to integrate the signal.

Samples were separated on a prepacked analytical column of Lichrospher 100 RP-18 5 μm (4-250 mm Merck; Merck, Sharpe & Dohme, Philadelphia, PA) protected by a 4 × 4 mm guard cartridge packed with the same matrix. The elution system consisted of 1% (v/v) acetic acid (A) and methanol (B) used in linear gradients of the following composition: 0-30 min: 0-15% B in A; 30-35 min: 15-60% B in A; 35-50 min: 60% B in A (isocratic). Separation was carried out at ambient temperatures at 1 ml/min and elution was monitored at 280 nm. Flavonoids were identified by comparison of UV spectra and retention times with those of purchased standards.

The following compounds were identified: quercetin 3-rhamnoside, (retention time (RT) 40.85)), quercetin 3-glucoside (RT 37.87), chlorogenic acid derivative (RT 18.49) and a polymer consisting of cinnamic acid glycoside (RT 18.67).

Example 7

BIOAUTOGRAPHIC ASSAYS ON THIN-LAYER-CHROMATOGRAMS

Flavonoid monomers extracted from Triumph, *ant*13-152, *ant*17-148 and *ant*18-159 were separated by HPLC (Fig. 1) and the individual fractions subjected to thin layer chromatography (TLC). 85% propanol was used as the solvent system, since it resulted in an acceptable separation of flavonoids and allowed fungal growth on the TLC plate after evaporation of the solvent. The method developed by Homans and Fuchs (1970) was adapted for detection of fungitoxic substances.

Each relevant HPLC fraction was divided into two aliquots: One sample was used for thin-layer chromatography (TLC) on Whatman TLC-cellulose plates (10 × 20 cm) which were developed with propanol-water (85:15% (v/v)) (Whatman Biosystems Ltd., Mainstone, Kent, UK). After drying overnight, TLC plates were sprayed with *F. culmorum* spore suspensions (5 ml, 2 × 10^6 spores/ml) in *Fusarium* nutrient solution (7 g KH_2PO_4, 3 g $NaHPO_4 \cdot 2H_20$, 4 g KNO_3, 1 g $MgSO_4 \cdot 7H_2O$, 1 g NaCl per 1 l ddH_2O and autoclaved) and 4.3% (w/v) glucose (sterile filtered). Plates were incubated for one week at room temperature.

The second sample of the purified flavonoid was applied onto the surface of a *Fusarium*-CZID plate and incubated as described under *Fusarium* inhibition assays above.

The results of a bioautographic assay and growth inhibition assays of HPLC fractions from the monomeric flavonoid separations are described below.

A TLC chromatogram of fraction 5 (RT 21.24 min), fraction 6 (RT 29.42 = catechin) and fraction 7 (RT 38.00 min = DHQ) from *ant*18-159, sprayed with 10^7 spores of *F. culmorum* was examined for signs of growth of *Fusarium*. Growth was detected 1 week post inoculation on the spot of fractions 5 and 6 but not on the DHQ-containing spot of fraction 7.

Seven individual HPLC fractions from the separation of the extract of *ant*13-152 spotted onto a CZID plate were incubated for 1 week after inoculation with 2 × 10^7 spores of *F. culmorum*. Retention times in min are for extracts utilized were as follows: fraction 1 = 5.42; 2 = 7.02; 3 = 9.01; 4 = 10.36; 5 = 15.48; 6 = 23.30; 7 = 38.54; 8 = 49.38. In examining the plate 1 week post infection, no inhibition zones were evident, *i.e.*, growth of Fusarium was detected uniformly on all areas of the plate.

10 individual HPLC fractions from the extract of *ant*18-159 were similarly examined 1 week post inoculation with 2 × 10^7 spores of *F. culmorum*. Of the 10 spots, only DHQ-containing spot 7 showed a marked inhibition zone. Retention times in minutes for extracts of *ant*18-159 were as

follows, where fractions correspond to spot number: fraction 1 = 7.18; 2 = 9.06; 3 = 12.24; 4 = 19.06; 5 = 21.24; 6 = 29.42; 7 = 38.00; 8 = 40.36; 9 = 45.12; and 10 = 65.06.

A brown color was observed in many HPLC fractions on the TLC plates where *Fusarium* grew. No inhibition was detected on TLC spots for isolated fractions of *ant*13-152 which lacks monomeric flavonoids and *ant*17-148, which accumulates large amounts of chrysoeriol. The fraction containing DHQ (RT 38 min) in the extract from mutant *ant*18-159 did not permit fungal growth nor sporulation on the TLC spot corresponding to DHQ.

For the second aliquots, no inhibition zones were detected for any of the applied fractions from *ant*13-152 or *ant*17-148. Catechin isolated from Triumph showed some degree of inhibition. A clear inhibition zone was seen for a fraction with a RT of 38 min purified from *ant*18-159. This antifungal fraction was identical to the one inhibiting growth on TLC plates. Standards, separated by HPLC under identical conditions, confirmed the identity of the active fraction as DHQ.

Example 8

ISOLATION AND CHARACTERIZATION OF BARLEY TESTA SPECIFIC PROMOTERS

Testa layers were manually isolated from barley caryopses between 10 and 30 days after anthesis. Awns and glumes were peeled off, whereafter the translucent epidermis layer, and the green pericarp (including the adhering chlorophyll/anthocyanin containing crosscell-layers) were removed. The testa layer was then separated from the aleurone layer.

A. Nucleic Acid Isolation

To obtain nucleic acids from isolated testa and aleurone tissue, the tissue was cut in small pieces in extraction buffer (500 mM NaCl, 50 mM Tris-HCl (pH 8.0), 50 mM EDTA, 1% (v/v) β-mercaptoethanol and 10% soluble polyvinylpolypyrrolidone (PVPP)). The tissue lines were repeatedly vacuum infiltrated, after which the tissue was ground in liquid nitrogen and extracted in the presence of insoluble PVPP extraction buffer and SDS.

After centrifugation at 15,000 rpm, residual polyphenolic compounds were removed from the supernatant on a PVPP column prior to phenol extraction. Poly-adenylated RNA was obtained in the procedure described in Kloppstech and Schweiger, 1976.

B. Difference Analysis

The representational difference analysis of Hubank and Schats (1994) was used to identify a gene that is expressed in wild type testa cells, but not in mutant *ant* 13 testa cells or wild type leaf

cells. Simplified cDNA versions of the mRNAs expressed in a cell isolate were generated by restriction enzyme digestion and PCR amplification.

If an amplifiable restriction fragment (the target) exists in one representation (wild type testa layer), and is absent from another (mutant *ant* 13 testa layer), an enrichment of the target can be obtained by subtractive hybridization of the tester (wild type) with an excess of driver (*ant*13).

With reference to Fig. 11, double-stranded cDNA of tester and driver was cut with a four base recognizing restriction enzyme (*Dpn*II), generating amplifiable fragments from 150 to 1000 bp. The single-stranded overhangs were ligated to a 24-mer oligonucleotide, while the recessed strand was elongated non-covalently with a 12-mer oligonucleotide, which base-pairs over 8 nucleotides to the 24-mer and with 4 nucleotides to the *Dpn*II overhang. The 12-mer part of this linker was then melted away and the single-stranded end of the fragment was filled in by the Taq DNA-polymerase to yield a fragment with the 24-mer ends, which can be removed with *Dpn*II.

These fragments are amplified by PCR with the 24-mer primer for the tester (wild type testa) and driver (mutant *ant* 13 testa) representations, respectively. The driver representation was digested with *Dpn*II, whereas the tester representation fragments are provided with a different 12/24 nucleotide linker. The two fragment populations were mixed in proportion of 1 tester to 100 drivers. With continued reference to Fig. 11, three types of hybrid molecules formed after a short denaturation at 98°C and annealing at 67°C for 20 hours. Driver-driver hybrids are eliminated during the subsequent amplification because of lack of primers. After melting out the 12-mer oligos and filling in the space with DNA polymerase, exponential amplification of the tester-tester hybrids (wildtype testa cDNA) with the 24 mer primer yielded "the first Difference Product" (DP1). During PCR amplification, the tester-driver hybrid yielded only linear amplified single strands, which were eliminated with mung bean nuclease.

"The first Difference Product" was provided with a new set of linkers and the selection procedures repeated until individual bands were visible after agarose gel electrophoresis and ethidium bromide staining of Difference Products 2 and 3.

C. Testa-Specific Promoter Clones

The two bands of DP3, designated RDA-1 and RDA-2, were cloned in BlueScript vectors and sequenced, yielding the sequences designated RDA-1 and RDA-2 (Figs. 13A and 13B, respectively). Total RNA from testa layers of wild type (Triumph) and mutant *ant*13 were isolated and analyzed for the presence of transcripts corresponding to the two Difference Products. Because of low abundance of the mRNA a combinatorial reverse transcription (RT) and PCR was employed to detect the transcript-specific cDNAs and identify the coding strand.

As illustrated in Fig. 8, the mRNA was reverse-transcribed with either oligo dT primers or primer 2 (oligonucleotide derived from sequence of Difference Product) and primer 1 (also oligonucleotide derived from the sequence of Difference Product (either RDA-1 or RDA-2). The obtained cDNA was then amplified by PCR. Only the combination of primers 1 and 2 or oligo-dT produced an amplificate. Based on Southern blot results, primer 1 (from RDA-1) in combination with oligo (dT) yielded a fragment 0.7 and 1 kbp in RNA from wild type testa/pericarp. If primer 2 was added as third primer to the assay, a fragment of the size as in the positive control was found. No signals were obtained using RNA from *ant*13 testa/pericarp and wild type (Triumph) leaf material. Therefore, tissue specificity for the corresponding transcript of RDA-1 was established.

RDA 2 was examined in similar experiments. If oligo (dT) or primer 2 was used for the reaction, a specific DNA fragment of expected size could be detected in the wild type RNA preparation, but not in the mutant *ant*13 or in wild type RNA preparations from the leaf.

A genomic library from the barley variety Igri in lambda fix II (Stratagene) was screened and genomic clones for the two RDA-clones were isolated. The genomic clone of RDA-1 has a size of ca. 13 kbp, the genomic clone of RDA-2 of ca. 15 kbp. A *Sac*I digest of both clones resulted in 3 major fragments: 6000, 3500 and 2000 bp for gRDA-1, and 6000, 3500 and 3000 bp for gRDA-2. If the two larger fragments are identical in the two clones, the two RDA fragments belong to the same gene. Sequencing confirmed the known RDA-2 fragments to be located at one end of the 3000 bp *Sac*I fragment. The remaining 2600 bp of the fragment were representing the upstream region of RDA-2, indicating that it contained the promoter of the gene in question.

Example 9

PRODUCTION OF A-ANTITRYPSIN IN FUNGAL-RESISTANT MALTED BARLEY SEEDS

A. Selectable Marker and AAT-Expression Transformation Vectors

A plasmid containing an α-antitrypsin gene under the control of an HV18 barley promoter and a signal sequence is constructed according to the procedures outlined in PCT patent application US94/13179, filed November 14, 1994, which is incorporated herein by reference. A selectable marker vector containing nucleic acid encoding the *bar* gene product is constructed by similar methods.

The two vectors are mixed at a molar ratio of target gene to selectable marker gene 1-6:1. 5-20 g of DNA is added to each tube and 20 μl of 0.1 M spermidine free base is added while vortexing. Slowly, in a dropwise fashion while vortexing, 50 μl of 2.5 M $CaCl_2$ is added. The solution is allowed to stand at room temperature for 10 minutes. The solution is centrifuged for 10 seconds and the supernatant is discarded. The particles are resuspended in 60 μl of 100% ethanol by

gently flicking the end of the tube. 10 μl of the particles are loaded onto one microcarrier as evenly as possible.

B. Plant Transformation

Ant18 barley mutants were obtained as above. Transformation of the mutant strain is carried out as described in Jensen, *et al.*, 1996, and in Wan, *et al.*, 1994, and as described above, using immature barley embryos. Typical rates of transformation are about 1% transformants per isolated immature zygotic embryo. Selection of transformed barley cells is on the herbicide phosphinothricin (Bialophos).

C. AAT Induction in Cell Culture

After selection of transgenic barley callus, callus cells are suspended in liquid culture containing AA2 media (Thompson, *et al.*, (1986), at 3% sucrose, pH 5.8. Thereafter, the cells are placed in multi-well tissue culture plates and shaken at 120 rpm in the dark for 48 hours. The supernatant is then removed and stored at -80°C prior to Western blot analysis of AAT. This procedure is employed to further select transgenic plant cells capable of producing the desired heterologous protein.

D. AAT Expression in Malted Seeds

The transgenic callus material from above is used to produce regenerated plants, according to standard methods. Seeds from the plants are harvested, and prepared for germination by an initial steeping step, in which the seeds immersed in or sprayed with water to increase the moisture content of the seed to between 35-45%. Steeping and germination are carried out as above.

E. Isolation of AAT

After optimum germination and expression of the AAT gene, the seeds are mashed (for example, by gentle grinding) to disrupt tissues and remove the hulls from the seeds. The seed mash is suspended in a protein extraction buffer, as above.

Supernatant obtained from the seeds is subjected to various purification schemes used in the wet-milling industry (*e.g.*, hydrocloning and ion exclusion chromatography) to remove un-wanted proteins and to concentrate AAT. Alternatively, ammonium sulfate precipitation can also be used to concentrate the AAT. Affinity- and ion-exchange chromatography can be used to purify the AAT away from other proteins in the supernatant. The presence of AAT in the various chromatographic fractions can be detected using standard photometric assays. Supernatants are concentrated using Centricon -10 filters (Amicon cat. #4207; Danvers, MA) and washed with induction media to remove

substances interfering with electrophoretic migration. Samples are concentrated approximately 10 fold. Concentrated samples are treated with an effective concentration of 1mM PMSF for 2-5 minutes to inactivate all serine protease activity.

Although the invention has been illustrated with respect to specific embodiments and examples, it will be appreciated that various changes and modification can be made without departing the invention as claimed.

SEQUENCE LISTING

(1) GENERAL INFORMATION

(i) APPLICANT: Applied Phytologics

(ii) TITLE OF THE INVENTION: Pathogen-Resistant Monocot Plants and Method

(iii) NUMBER OF SEQUENCES: 7

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: Dehlinger & Associates
(B) STREET: 350 Cambridge Ave. Suite 250
(C) CITY: Palo Alto
(D) STATE: CA
(E) COUNTRY: USA
(F) ZIP: 94306

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Diskette
(B) COMPUTER: IBM Compatible
(C) OPERATING SYSTEM: DOS
(D) SOFTWARE: FastSEQ for Windows Version 2.0

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(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Evans, Susan T
(B) REGISTRATION NUMBER: 38,443
(C) REFERENCE/DOCKET NUMBER: 0665-0013.41

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: 650-324-0880
(B) TELEFAX: 650-324-0960
(C) TELEX:

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 3193 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 4467 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

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GATCCTCAAG CAGGTGCAGC TCATCCACCT CGACGACCTC TGCGACGCCG AGATCTTCCT 2940
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CGGCCTCGCC GCCATGCTCA GGGATAGGTA CCCCGAGTAC GACGTCCCGC AGAGGTTCCC 3060
CGGGATCCAG GACGACCTCC AGCCGGTGCG CTTCTCGTCC AAGAAGCTCC AGGACCTCGG 3120
GTTCACCTTC AGGTACAAGA CGCTGGAGGA CATGTTCGAC GCCGCCATCC GGACTTGCCA 3180
GGAGAAGGGC CTCATCCCCC TCGCCACTGC CGCCGGAGGG GACGGCTTTG CCTCGGTGCG 3240
CGCACCCGGC GAGACGGAGG CGACGATTGG CGCTTAAGCA ACAATCACCC GGCTCTCCCC 3300
GTCGATGATG CTATCAGCTA TCTATCTCTT CGAGCTCCGC TGCCAATACG GAACTTACTT 3360
CCATGTTCAA CGAATTGTTT TATCCCCTGG ATTACGTATT GTTTTTATGT TTTATACCCA 3420
AACAACGAAT GTTTCTCAGT ATTTTACTAC AGCTAGCCCC TATAGTAGAG ATTCTATTAT 3480
ATGAATAAGC TATCTGTAGA GGCTCTGGTG CAATATATAT TTCGAGTTAT TTGGATTACT 3540
AGCATAATTC AATTTTTAAA AACTATTATT ATTATTCACT TATTCGGGAG GCTACTATTA 3600

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TTAGGTTTTT TCTTTTTTTG GGATCCAATA ACTCCATGCA TATTTTTTCT TTTTTTTGCA   3840
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CGTAGAACAA CCGAACTTTT CTCCAGTAAC AATCGGGACG TTTCGTCGGT AATAACCGAA   4320
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(A) LENGTH: 1438 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

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CTGTGGAAGG CCGACCTGGG CGAGGAAGGC AGCTTCGACG CGGCGATCAG GGGTTGCACG    360
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GTCAAGCGCA TCGTCTTCAC CTCCTCCGCC GGGACCGTCA ACATCGAGGA GCGGCAGCGC    540
CCCTCCTACG ACCACGACGA CTGGAGCGAC ATCGACTTCT GTCGCCGCGT CAAGATGACC    600
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GAGCACGGGC TGGACCTCAT CAGCGTCATC CCCACGCTCG TCGTCGGGCC CTTCATCAGC    720
AACGGGATGC CGCCGAGCCA CGTCACCGCG CTGGCGCTGC TCACGGGGAA CGAGGCCCAC    780
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(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 582 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

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GTCCTGCGTC GTCGCCGCTC GCAGGCGGTG GTACGGTGCG TGCAGTGCAT CCAATAATTC 180
CATCGATCGG TAGCAGCAGC CAAAAACTTT GATTCGCTCC GTGTCGTCGT GTTCATTCTC 240
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(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 650 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

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(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 469 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

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AACAAACTCC TACCAATCAG AAATGGCATA AACCCACATC CAAAGTGGTT AAGGCCCGGT 360
TTTATGAAAT GTCTTCAAAG TGGCAAAGCT AGGAGTATTA TAACTTGATG AGTATTAAGA 420
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(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 401 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

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AGATGTGTTG AAGCGATGAT ACCTTTTCCA CGCGCAGCCC TGCTAGTCCT GAGTGGCTCG 360
GCTCTACGGC CCGGTCCGGA TAGGTCATCA CAACCTTGAT C 401

IT IS CLAIMED:

1. A method of producing resistance to a plant microbial pathogen in the seeds of a monocot plant, comprising

identifying an intermediate secondary metabolite that can be shown to inhibit infection of the plant pathogen in seeds, at above-normal levels of the metabolite,

identifying a gene encoding an enzyme that normally converts the intermediate secondary metabolite to another metabolic that is less toxic to the pathogen, and

stably transforming a monocotyledonous plant with a chimeric gene having (i) a transcriptional regulatory region induced during seed development, and (ii) an inhibitory gene sequence operably linked to said transcriptional regulatory region for induction of an inhibitory transcript RNA capable of inhibiting expression of the identified gene.

2. The method of claim 1, wherein the plant is selected from the group consisting of barley, rice, wheat, and corn.

3. The method of claim 1, wherein said inhibitory transcript RNA is capable of sequence-specific hybridization with the mRNA transcript of the identified plant gene.

4. The method of claim 3, wherein the pathogen is a fungal pathogen, and said secondary metabolite is a flavonoid compound.

5. The method of claim 4, wherein the pathogen is *Fusarium*, said metabolite is dihydroquercetin, and the identified gene encodes a dihydroquercetin reductase (DHQR) enzyme capable of converting dihydroquercetin to 2,3-trans-3,4,-cis-leucocyanidin.

6. The method of claim 5, wherein the monocot is barley, and the transcript RNA is capable of sequence-specific hybridization to the transcript of the structural gene for barley DHQR, identified by SEQ ID NO:1.

7. The method of claim 5, wherein the monocot is corn, and the transcript RNA is capable of sequence-specific hybridization to the transcript of the structural gene for corn DHQR, identified by SEQ ID NO:2.

8. The method of claim 1, wherein the monocot is barley, and the transcriptional regulatory region is a barley-seed tissue-specific or testa-specific promoter selected from the group consisting of a barley (1-3, 1-4)-β-glucanase isoenzyme I gene promoter, a barley (1-3, 1-4)-β-glucanase isoenzyme II gene promoter, a barley B22E gene promoter, a barley B-hordein gene promoter, a barley B1 hordein gene promoter, a barley C-hordein gene promoter, a barley β-amylase gene promoter, and a barley "DP3" gene promoter.

9. The method of claim 1, wherein the monocot is corn, and the transcriptional regulatory region is a corn-kernel tissue-specific or testa-specific promoter selected from the group consisting of a corn *O2*-opaque2 gene promoter, a corn *Sh2*-shrunken 2 gene promoter, a corn Bt2-brittle 2 gene promoter, a corn *Agp1* gene promoter, a corn *Agp2* gene promoter, and a corn *Zp1* zein gene promoter.

10. The method of claim 1, wherein the monocot is rice, and the transcriptional regulatory region is a rice-grain tissue-specific or testa-specific promoter selected from the group consisting of a rice Gt1 gene promoter, a rice Gt2 gene promoter, a rice Gt3 gene promoter, a rice GluB-3 gene promoter, a rice GluB-2 gene promoter, a rice GluB-1 gene promoter, and a rice GluA-3 gene promoter.

11. A monocotyledonous plant which is stably transformed with a chimeric gene having (i) a transcriptional regulatory region induced during seed development, and (ii) an inhibitory gene sequence operably linked to said transcriptional regulatory region for induction of an inhibitory transcript RNA during seed development, where the transcript RNA is capable of inhibiting expression of an identified gene whose gene product is an enzyme that normally converts a selected intermediate secondary metabolite that is toxic to the pathogen to another metabolic that is less toxic to the pathogen.

13. The plant of claim 12, wherein said plant is selected from the group consisting of barley, rice, corn, and wheat.

14. The plant of claim 12, wherein said inhibitory transcript RNA is capable of sequence-specific hybridization with the transcript of identified gene.

15. The plant of claim 14, which has enhanced resistance to fusarium infection, wherein the product of said identified plant gene is a DHQR enzyme capable of reducing dihydroquercetin to 2,3-trans-3,4-cis-leucocyanidin.

16. The plant of 15, which is barley, wherein the inhibitory transcript RNA is capable of sequence-specific hybridization to the transcript of the structural gene for barley DHQR, identified by SEQ ID NO:1.

17. The plant of claim 15, wherein the monocot is corn, and the inhibitory transcript RNA is capable of sequence-specific hybridization to the transcript of the structural gene for corn DHQR, identified by SEQ ID NO:2.

18. The plant of claim 12, wherein the monocot is barley, and the transcriptional regulatory region is a barley-seed tissue-specific or testa-specific promoter selected from the group consisting of a barley (1-3, 1-4)-β-glucanase isoenzyme I gene promoter, a barley (1-3, 1-4)-β-glucanase isoenzyme II gene promoter, a barley B22E gene promoter, a barley B-hordein gene promoter, a barley B1 hordein gene promoter, a barley C-hordein gene promoter, a barley β-amylase gene promoter, and a barley "DP3" gene promoter.

19. The plant of claim 12, wherein the monocot is corn, and the transcriptional regulatory region is a corn-kernel tissue-specific or testa-specific promoter selected from the group consisting of a corn *O2*-opaque2 gene promoter, a corn *Sh2*-shrunken 2 gene promoter, a corn Bt2-brittle 2 gene promoter, a corn *Agp1* gene promoter, a corn *Agp2* gene promoter, and a corn *Zp1* zein gene promoter.

20. The plant of claim 12 wherein the monocot is rice, and the transcriptional regulatory region is a rice-grain tissue-specific or testa-specific promoter selected from the group consisting of a rice Gt1 gene promoter, a rice Gt2 gene promoter, a rice Gt3 gene promoter, a rice GluB-3 gene promoter, a rice GluB-2 gene promoter, a rice GluB-1 gene promoter, and a rice GluA-3 gene promoter.

21. A monocot seed produced by the plant of claim 12.

22. The monocot seed of claim 21, which is a barley or corn seed, and the inhibitory transcript RNA is capable of sequence-specific binding to a dihydroquercetin reductase (DHQR) gene

whose sequence is selected from the group consisting of SEQ ID NO:1 and SEQ ID NO:2, respectively.

23. The seed of claim 21, wherein the transcriptional regulatory region is a seed tissue-specific or testa-specific promoter.

24. A method of producing a therapeutic heterologous protein substantially free of mycotoxins, comprising

genetically altering a monocot plant to achieve elevated seed levels of a secondary metabolite effective to inhibit fungal infection of seeds from the plant,

stably transforming the plant with a chimeric gene having (i) a transcriptional regulatory region inducible during seed germination, (ii) a first DNA sequence encoding the heterologous protein, and (iii) a second DNA sequence encoding a signal polypeptide, where said second DNA sequence is operably linked to said transcriptional regulatory region and said first DNA sequence, and where said signal polypeptide is in translation-frame with said protein and is effective to facilitate secretion of said protein across the aleurone or scutellar epithelium layer of seeds from the altered, transformed plant into the endosperm,

obtaining seeds from the altered, transformed plant,

malting said seeds under conditions favoring heterologous protein production in the seeds, and

isolating said therapeutic heterologous protein from the seeds.

25. The method of claim 32, where said altering comprises mutating a plant gene encoding an enzyme that converts said secondary metabolite to another product that is less toxic to the fungus, to produce a mutant gene incapable of producing said enzyme in the plant.

26. The method of claim 33, where said monocot plant is barley and said gene is an *ant*18 mutant.

27. The method of claim 33, where said plant is corn and said gene is a corn A1 gene mutant.

28. The method of claim 33, where said plant is rice and said gene is a rice A1 gene mutant.

29. The method of claim 32, wherein said altering comprises transforming the monocot plant with a chimeric gene having (i) a transcriptional regulatory region induced during seed development,

and (ii) an inhibitory gene sequence operably linked to said transcriptional regulatory region for induction of an inhibitory transcript RNA capable of inhibiting expression of a gene encoding an enzyme that normally converts the intermediate secondary metabolite to another metabolic that is less toxic to said fungus.

30. The method of claim 37, wherein said fungus is *Fusarium* and said inhibitory transcript RNA is capable of inhibiting expression of a DHQR gene.

31. The method of claim 38, wherein the monocot is barley, and the transcript RNA is capable of sequence-specific hybridization to the transcript of the structural gene for barley DHQR, identified by SEQ ID NO:1.

32. The method of claim 38, wherein the monocot is corn, and the transcript RNA is capable of sequence-specific hybridization to the transcript of the structural gene for corn DHQR, identified by SEQ ID NO:2.

Fig. 1A

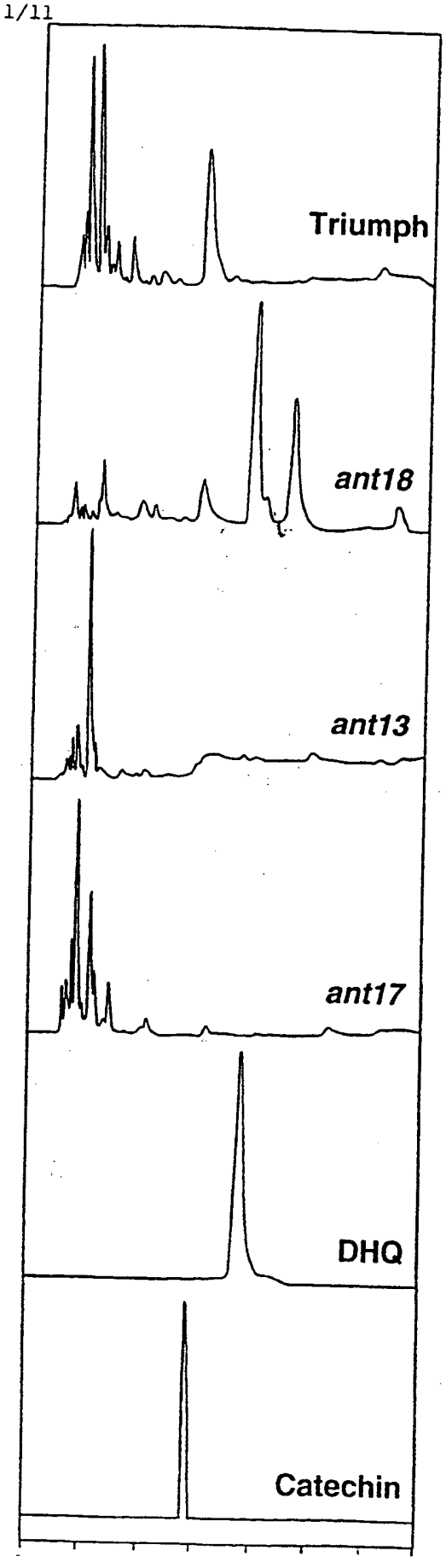
Fig. 1B

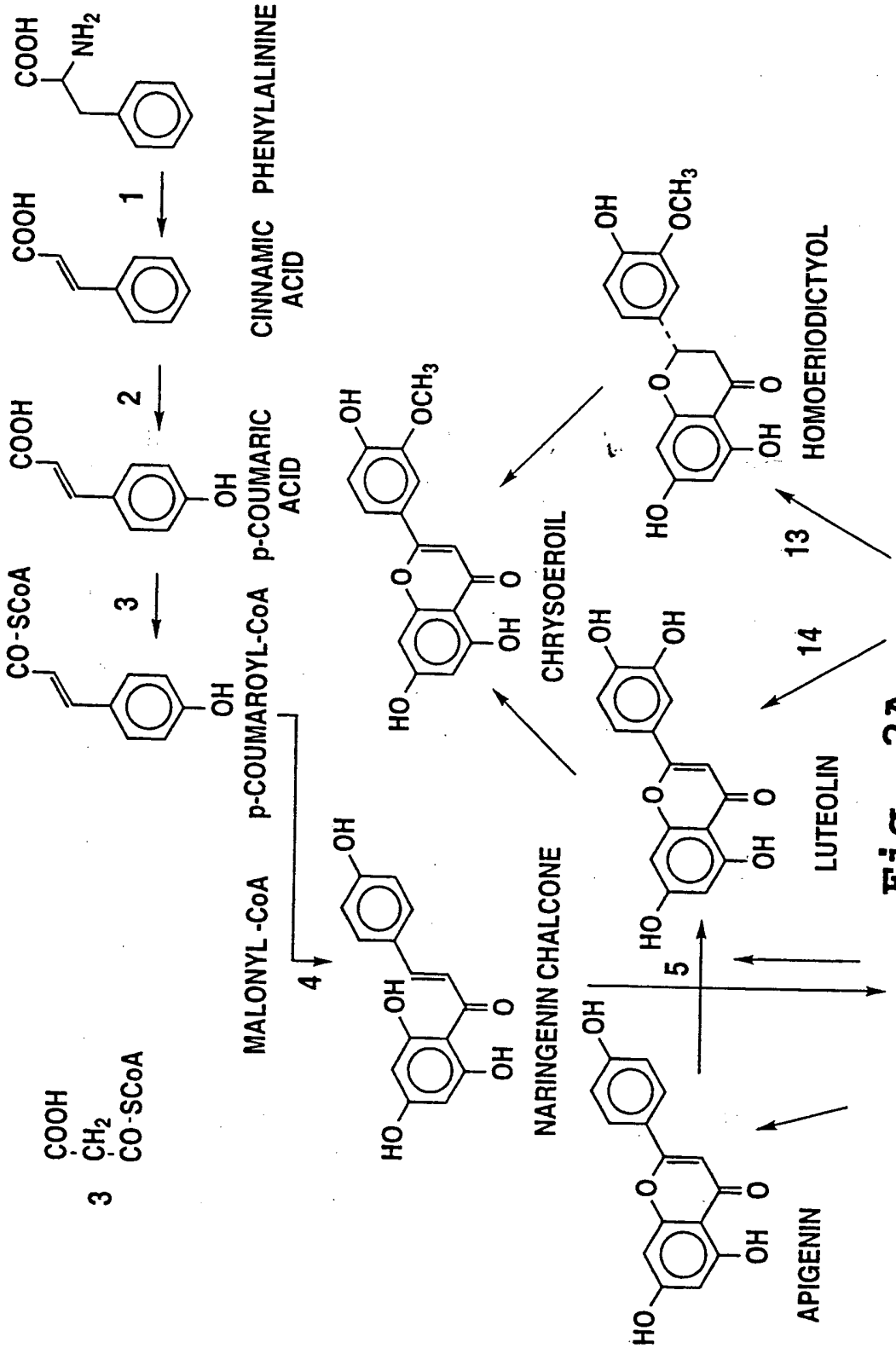
Fig. 1C

Fig. 1D

Fig. 1E

Fig. 1F

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APIGENIN
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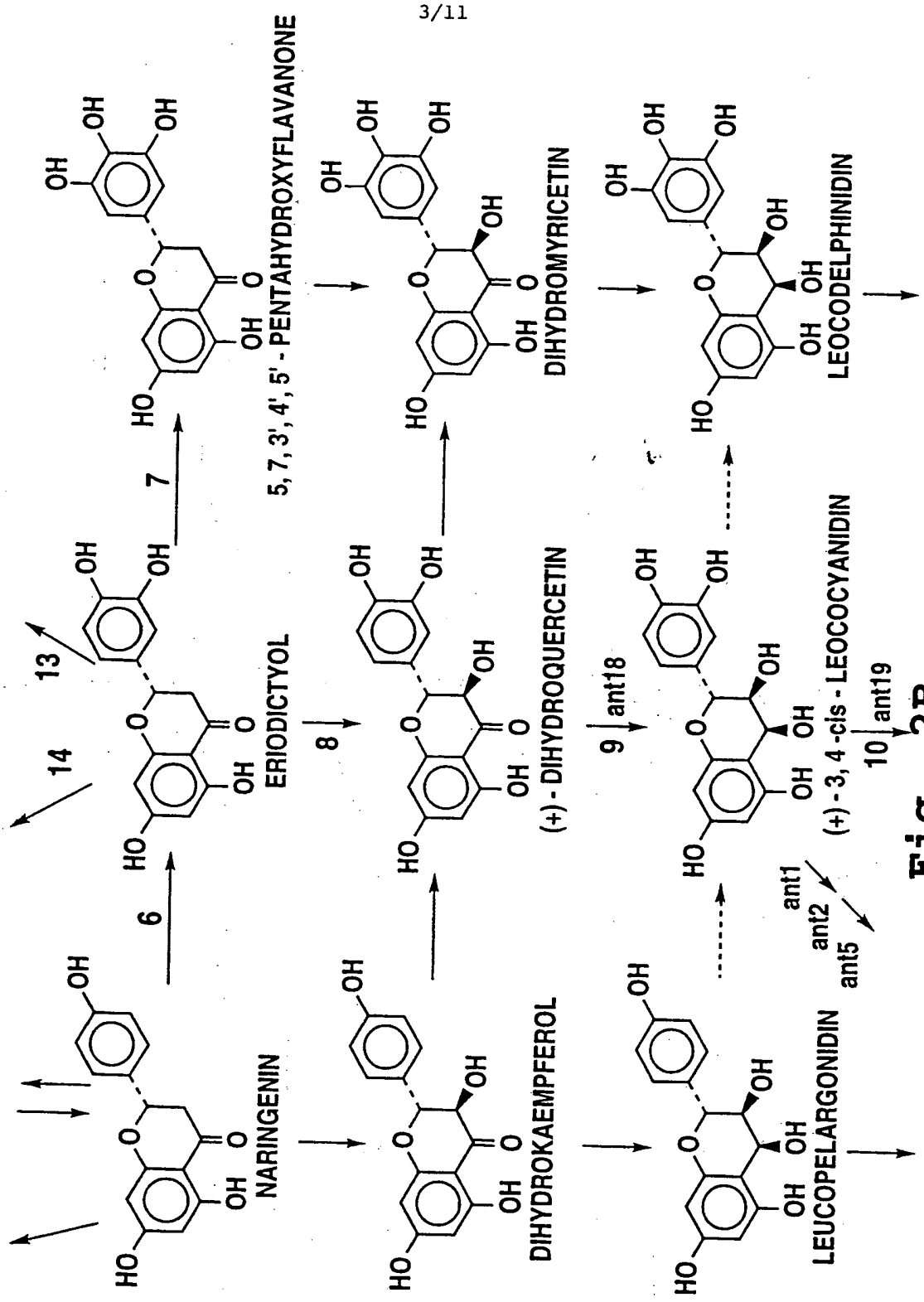
2
3
4
5
13
14

Fig. 2A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(+) - DIHYDROQUERCETIN
DIHYDROMYRICETIN
LEUCOPELARGONIDIN
(+) - 3, 4 - cis - LEOCOCYANIDIN
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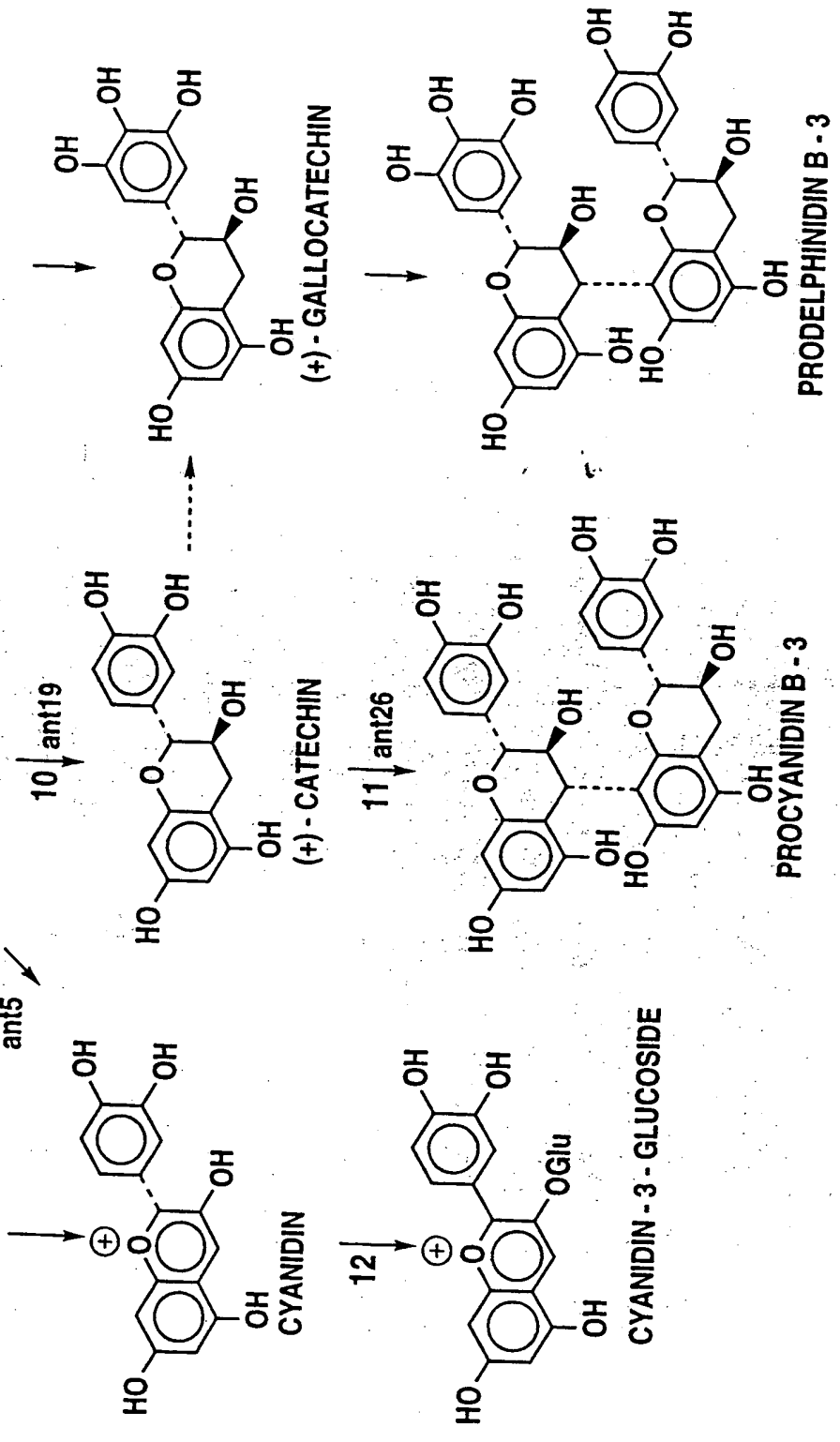
7
8
9
10
13
14
ant1
ant2
ant5
ant18
ant19

Fig. 2B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ant2
ant5
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10
ant19
(+) - CATECHIN
(+) - GALLOCATECHIN
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ant26
PROCYANIDIN B - 3
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HO

Fig. 2C

Fig. 3A

Fig. 3B

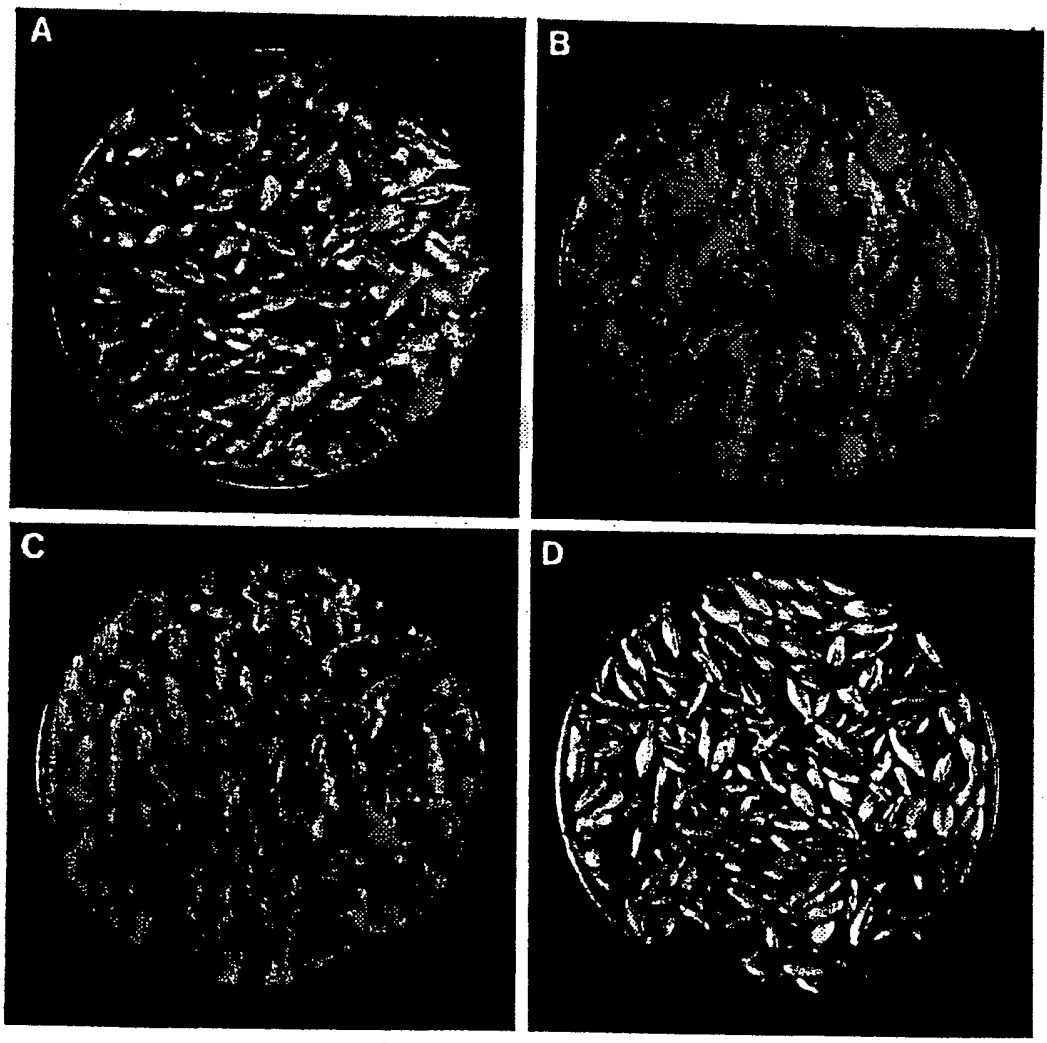

Fig. 3C

Fig. 3D

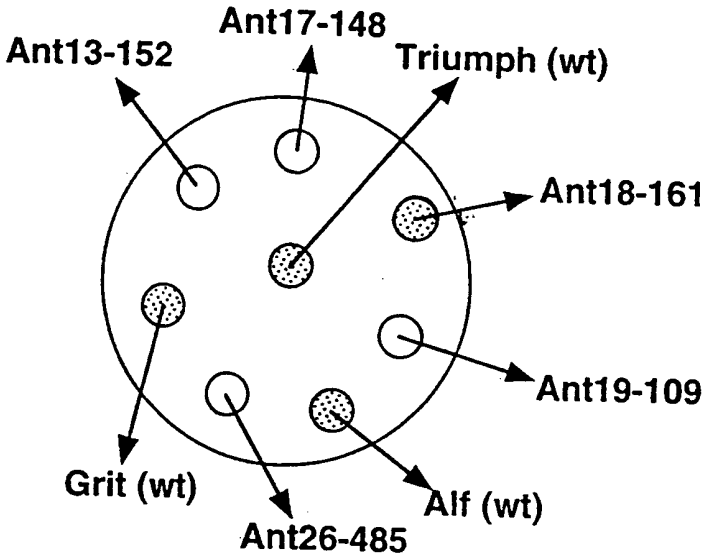
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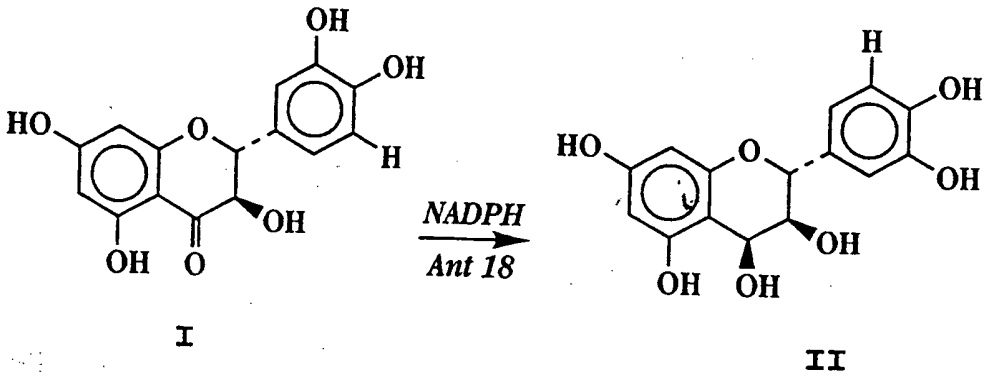
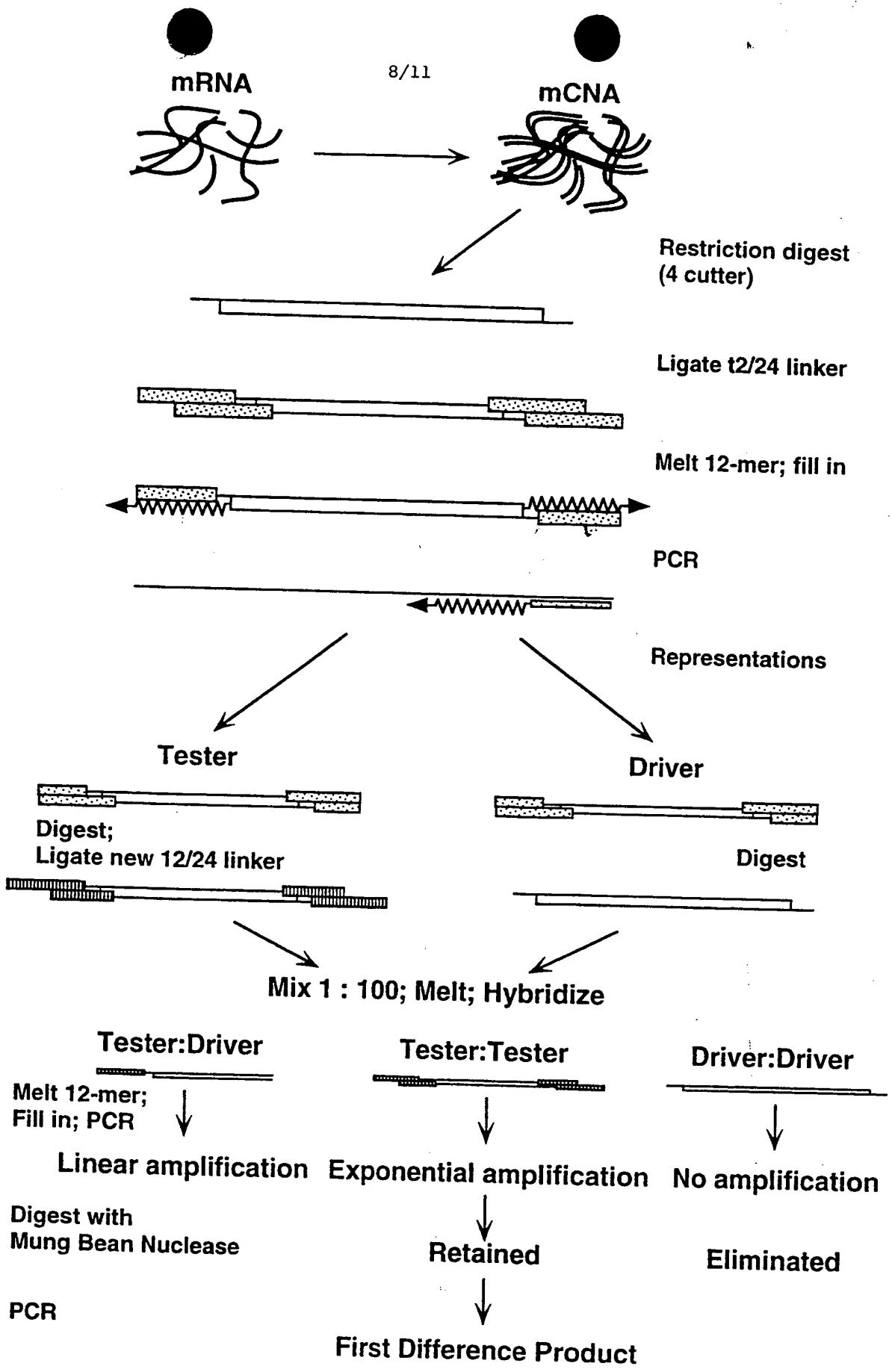
Fig. 4



I II

Fig. 5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mCNA
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(4 cutter)
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Melt 12-mer; fill in
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Representations
Tester
Driver
Digest;
Ligate new 12/24 linker
Digest
Mix 1 : 100; Melt; Hybridize
Tester:Driver
Tester:Tester
Driver:Driver
Melt 12-mer;
Fill in; PCR
Linear amplification
Exponential amplification
No amplification
Digest with
Mung Bean Nuclease
Retained
Eliminated
PCR
First Difference Product

Fig. 6

(a)

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Fig. 7A

(b)

```
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190         200         210         220         230         240
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250         260         270         280         290         300
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Fig. 7B

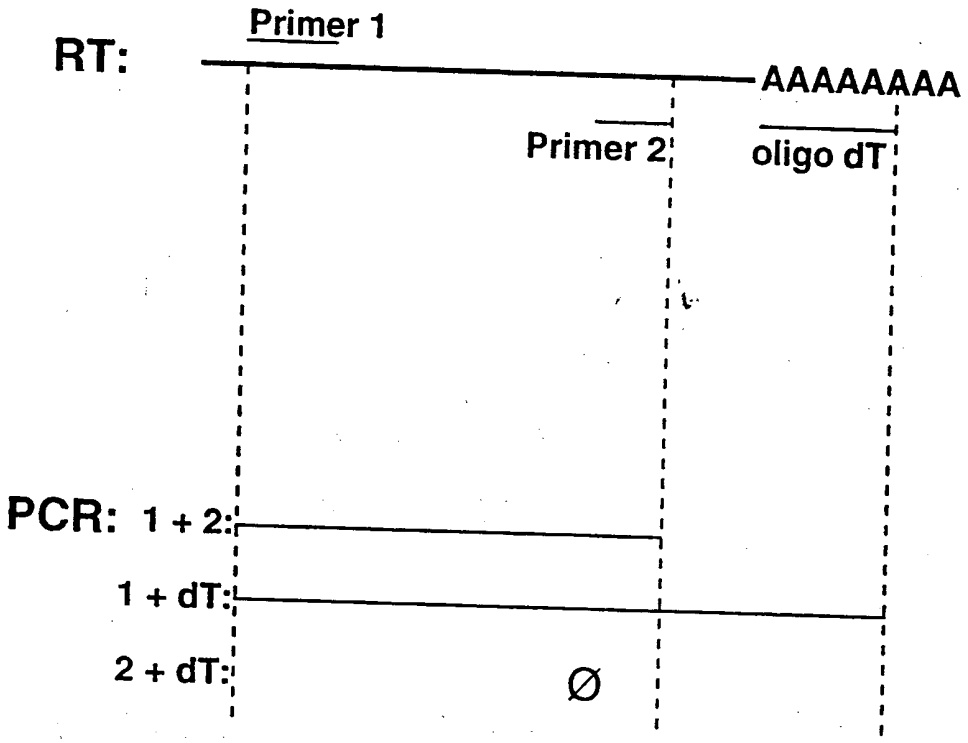

Fig. 8

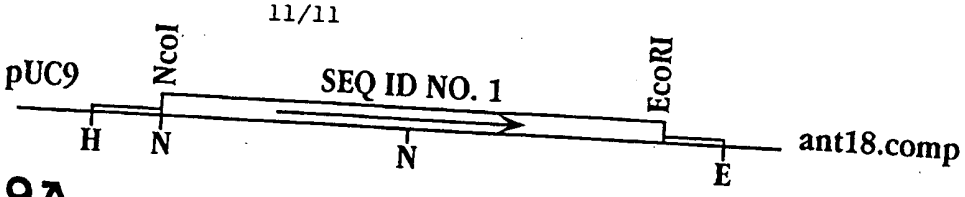

Fig. 9A

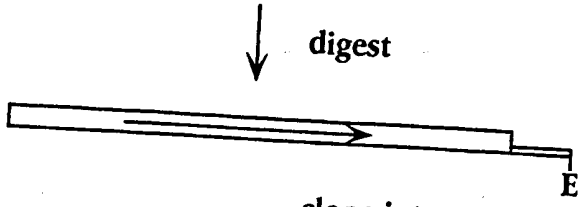

Fig. 9B

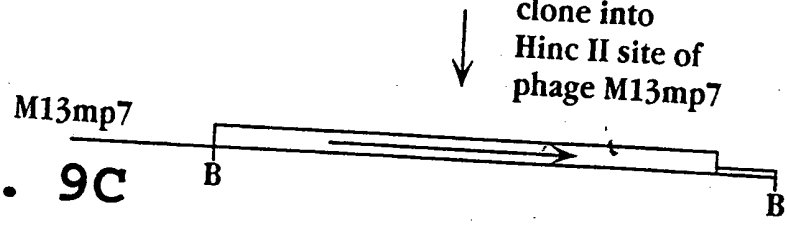

Fig. 9C

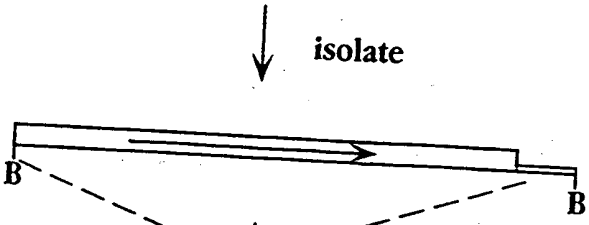

Fig. 9D

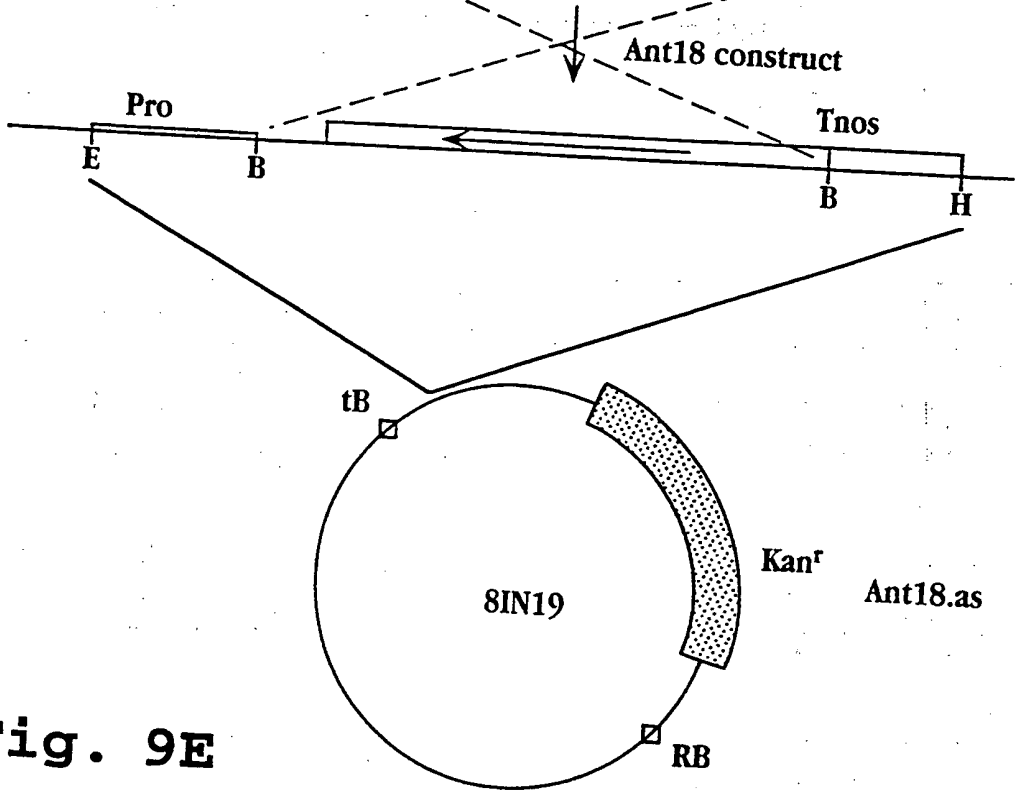

Fig. 9E

INTERNATIONAL SEARCH REPORT

Intern: al Application No
PCT/US 98/12917

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/53 C12N15/11 C12N15/82 A01H5/00 A01H5/10

According to International Patent Classification(IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N C07K A01H

Documentation searched other than minimumdocumentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and. where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication. where appropriate. of the relevant passages	Relevant to claim No.
X	WO 96 41528 A (PROGUARD INC ;EMERSON RALPH W (US); CRANDALL BRADFORD G JR (US)) 27 December 1996 * see the whole document, esp. p.23-25; example 14 *	1-4, 8-14, 18-21,23
X A	WO 96 20595 A (PROGUARD INC ;EMERSON RALPH W (US); CRANDALL BRADFORD G JR (US)) 11 July 1996 * see esp. p.16 l.31 - p.24. l.19; example 9 *	1-4, 8-14, 18-21,23 24-32

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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Date of the actual completion of theinternational search	Date of mailing of the international search report
20 October 1998	02/11/1998

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Kania, T

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International Application No
PCT/US 98/12917

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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A	WO 95 18859 A (CIBA GEIGY AG ;UNIV RESEARCH CORP (US); ROBERTS WALDEN K (US); SEL) 13 July 1995 see the whole document	1-32
A	WANG X. ET AL.: "Expression of the dihydroflavonol reductase gene in an anthocyanin-free barley mutant" HEREDITAS, vol. 119, no. 1, 1993, pages 67-75, XP002081491 see the whole document	1-32
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A	HAMILL, J. D. ET AL: "Manipulating secondary metabolism in culture" , BIOSYNTHESIS AND MANIPULATION OF PLANT PRODUCTS, (1993) PP. 178-209. PLANT BIOTECHNOLOGY VOL. 3. 7 PP. OF REF. PUBLISHER: BLACKIE ACADEMIC & PROFESSIONAL. GLASGOW ISBN: 0-7154-0060-2 XP002081493 * see esp. chapters 5.4-5.6 *	1-32

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Internal Application No
PCT/US 98/12917

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